

Contents lists available at [ScienceDirect](http://www.sciencedirect.com)

# Toxicology in Vitro

journal homepage: [www.elsevier.com/locate/toxinvit](http://www.elsevier.com/locate/toxinvit)

## Review

### Report from the EPAA workshop: *In vitro* ADME in safety testing used by EPAA industry sectors

K. Schroeder<sup>a,u</sup>, K.D. Bremm<sup>b,\*</sup>, N. Alépée<sup>c</sup>, J.G.M. Bessems<sup>d</sup>, B. Blaauboer<sup>e</sup>, S.N. Boehn<sup>f</sup>, C. Burek<sup>g</sup>, S. Coecke<sup>h</sup>, L. Gombau<sup>i</sup>, N.J. Hewitt<sup>j</sup>, J. Heylings<sup>k</sup>, J. Huwyler<sup>l</sup>, M. Jaeger<sup>m</sup>, M. Jagelavicius<sup>n</sup>, N. Jarrett<sup>o</sup>, H. Ketelslegers<sup>p</sup>, I. Kocina<sup>q</sup>, J. Koester<sup>r</sup>, J. Kreysa<sup>h</sup>, R. Note<sup>c</sup>, A. Poth<sup>m</sup>, M. Radtke<sup>s</sup>, V. Rogiers<sup>t</sup>, J. Scheel<sup>u</sup>, T. Schulz<sup>v</sup>, H. Steinkellner<sup>w</sup>, M. Toeroek<sup>x</sup>, M. Whelan<sup>h</sup>, P. Winkler<sup>y</sup>, W. Diembeck<sup>z</sup>

<sup>a</sup> BioMed – zet Life Science GmbH, zet Life Science Laboratorium, Industriezeile 36/VII, A-4020 Linz, Austria<sup>b</sup> Bayer HealthCare AG, D-42096 Wuppertal, Germany<sup>c</sup> L'Oréal – Life Sciences, 1, Avenue Eugène Schueller, 93601 Aulnay Sous Bois Cedex, France<sup>d</sup> National Institute of Public Health and the Environment (RIVM), Bilthoven, The Netherlands<sup>e</sup> Institute for Risk Assessment Sciences (IRAS), Utrecht University, P.O. Box 80.176, 3508 TD Utrecht, The Netherlands<sup>f</sup> BASF, Carl-Bosch-Strasse 38, 67056 Ludwigshafen, Germany<sup>g</sup> University of Wurzburg, Cronthalstr. 6A, 97074 Wurzburg, Germany<sup>h</sup> ECVAM-European Centre for the Validation of Alternative Methods, Institute for Health and Consumer Protection, European Commission, Joint Research Center, Ispra, Italy<sup>i</sup> Advancell, In Vitro Cell Technologies, Centro de Investigación, Avda. Campanar 21, Hospital La Fe 46009-Valencia, Spain<sup>j</sup> SWS, Wingertstrasse 25, 64390 Erzhhausen, Germany<sup>k</sup> Dermal Technology Laboratory (DTL Ltd.), Keele University Science Park, United Kingdom<sup>l</sup> University of Applied Sciences (FHNW), School of Life Sciences, Department of Pharma Technology, Gründenstrasse 40, CH-4132 Muttenz, Switzerland<sup>m</sup> Harlan Cytotest Cell Research, 64380 Rosdorf, Germany<sup>n</sup> Lithuanian State Plant Protection, Vilnius, Lithuania<sup>o</sup> European Medicines Agency, London, UK<sup>p</sup> ExxonMobil Petroleum & Chemical, Machelen, Belgium<sup>q</sup> Food and Veterinary Service of the Republic of Latvia, Riga, Latvia<sup>r</sup> Bayer Crop Science AG, Monheim/Rhein, Germany<sup>s</sup> Bayer Healthcare AG, 1 Kaiser-Wilhelm-Allee, 51373 Leverkusen, Germany<sup>t</sup> Free University of Brussel, Brussels, Belgium<sup>u</sup> Henkel AG & Co. KGaA, Henkelstr. 67, 40589, Düsseldorf, Germany<sup>v</sup> Federal Institute of Risk Assessment, Berlin, Germany<sup>w</sup> European Food Safety Authority, Parma, Italy<sup>x</sup> Actelion Pharmaceuticals Ltd, Gewerbestrasse 16, CH-4123 Allschwil, Switzerland<sup>y</sup> Johnson & Johnson GmbH, Johnson & Johnson Platz 2, 41470 Neuss, Germany<sup>z</sup> Beiersdorf AG, Unnastraße 48, 20253 Hamburg, Germany

**Abbreviations:** ADME, absorption, distribution, metabolism, and excretion; ADI, Acceptable Daily Intake; Colipa, the European Cosmetic Toiletry and Perfumery Association; CYPs, cytochrome P450s; DNEL, Derived No Effect Level; DG, Directorate General; ECHA, EU Chemicals Agency; ECVAM, European Centre for the Validation of Alternative Methods; EFSA, European Food Safety Authority; EU, European Union; FP6, 6th Framework Programme; FP7, 7th Framework Programme; GSTs, glutathione S-transferases; GLP, Good Laboratory Practice; GCCP, Good Cell Culture Practice; IMI, Innovative Medicines Initiative; ICH, International Conference on Harmonisation; IND, Investigational New Drug; ITS, Integrated Testing Strategies; JECFA, Joint FAO/WHO Expert Committee on Food Additives; JMPR, Joint FAO/WHO Expert on Pesticide Residues; JTIs, Joint Technology Initiatives; MoS, Margin of Safety; NRC, National Research Council; NIH, National Institutes of Health; NOAEL, No Observed Adverse Effect Level; OECD TG, Organisation for Economic Co-operation and Development Technical Guideline; OSIRIS, Optimised Strategies for Risk Assessment of Industrial Chemicals through Integration of Non-Test and Test Information; PBBK, physiologically-based biokinetic; PBPK, physiologically-based pharmacokinetic; PBTK, physiologically-based toxicokinetic; PPPs, plant protection products; PPR, Plant Protection Products and their Residues; QPS, Qualified Presumption of Safety; QSAR, Quantitative Structure Activity Relationship; REACH, Registration, Evaluation, Authorisation and Restriction of Chemicals; SCCNFP, Scientific Committee on Cosmetics and Non-Food Products intended for consumers; SCCS, Scientific Committee on Consumer Safety; SCCP, Scientific Committee on Consumer Products; SULTs, sulfotransferases; TTC, Threshold of Toxicological Concern; TK, toxicokinetics; UGTs, UDPGA-glucuronosyltransferases; FDA, US Food and Drug Administration; WHO, World Health Organization.

\* Corresponding author. Address: Bayer Schering Pharma AG, BSP-GDD-GED-AM, D-42096 Wuppertal, Germany. Tel.: +49 202 36 8019; mobile: +49 1753108019; fax: +49 202 36 8233.

E-mail addresses: [klaus-dieter.bremm@bayer.com](mailto:klaus-dieter.bremm@bayer.com) (K.D. Bremm), [NALEPEE@rd.loreal.com](mailto:NALEPEE@rd.loreal.com) (N. Alépée), [jos.bessems@rivm.nl](mailto:jos.bessems@rivm.nl) (J.G.M. Bessems), [b.blaauboer@uu.nl](mailto:b.blaauboer@uu.nl) (B. Blaauboer), [susanne.boehn@basf.com](mailto:susanne.boehn@basf.com) (S.N. Boehn), [christof.burek@mail.uni-wuerzburg.de](mailto:christof.burek@mail.uni-wuerzburg.de) (C. Burek), [sandra.coecke@jrc.it](mailto:sandra.coecke@jrc.it) (S. Coecke), [lourdes.g@advancell.net](mailto:lourdes.g@advancell.net) (L. Gombau), [nickyhewitttd@yahoo.co.uk](mailto:nickyhewitttd@yahoo.co.uk) (N.J. Hewitt), [j.heyling@dermaltechnology.com](mailto:j.heyling@dermaltechnology.com) (J. Heylings), [joerg.huwyler@fhnw.ch](mailto:joerg.huwyler@fhnw.ch) (J. Huwyler), [mjaeger@harlan.com](mailto:mjaeger@harlan.com) (M. Jaeger), [vaatmj@vaat.lt](mailto:vaatmj@vaat.lt) (M. Jagelavicius), [nicholas.jarrett@ema.europa.eu](mailto:nicholas.jarrett@ema.europa.eu) (N. Jarrett), [hans.ketelslegers@exxonmobil.com](mailto:hans.ketelslegers@exxonmobil.com) (H. Ketelslegers), [iveta.kocina@pvd.gov.lv](mailto:iveta.kocina@pvd.gov.lv) (I. Kocina), [johannes.koester@bayercropscience.com](mailto:johannes.koester@bayercropscience.com) (J. Koester), [Joachim.Kreysa@ec.europa.eu](mailto:Joachim.Kreysa@ec.europa.eu) (J. Kreysa), [rnote@rd.loreal.com](mailto:rnote@rd.loreal.com) (R. Note), [poth@harlan.com](mailto:poth@harlan.com) (A. Poth), [martin.radtke@bayerhealthcare.com](mailto:martin.radtke@bayerhealthcare.com) (M. Radtke), [vrogiers@vub.ac.be](mailto:vrogiers@vub.ac.be) (V. Rogiers), [julia.scheel@henkel.com](mailto:julia.scheel@henkel.com) (J. Scheel), [Thomas.Schulz@bfr.bund.de](mailto:Thomas.Schulz@bfr.bund.de) (T. Schulz), [johann.steinkellner@efsa.europa.eu](mailto:johann.steinkellner@efsa.europa.eu) (H. Steinkellner), [michael.toeroek@dsm.com](mailto:michael.toeroek@dsm.com) (M. Toeroek), [Maurice.Whelan@jrc.ec.europa.eu](mailto:Maurice.Whelan@jrc.ec.europa.eu) (M. Whelan), [PWinkle2@its.jnj.com](mailto:PWinkle2@its.jnj.com) (P. Winkler), [walter.diembeck@beiersdorf.com](mailto:walter.diembeck@beiersdorf.com) (W. Diembeck).

## ARTICLE INFO

## Article history:

Received 25 June 2010

Accepted 6 December 2010

Available online 15 December 2010

## Keywords:

EPAA workshop

*In vitro*

ADME

Safety testing

## ABSTRACT

There are now numerous *in vitro* and *in silico* ADME alternatives to *in vivo* assays but how do different industries incorporate them into their decision tree approaches for risk assessment, bearing in mind that the chemicals tested are intended for widely varying purposes? The extent of the use of animal tests is mainly driven by regulations or by the lack of a suitable *in vitro* model. Therefore, what considerations are needed for alternative models and how can they be improved so that they can be used as part of the risk assessment process? To address these issues, the European Partnership for Alternative Approaches to Animal Testing (EPAA) working group on prioritisation, promotion and implementation of the 3Rs research held a workshop in November, 2008 in Duesseldorf, Germany. Participants included different industry sectors such as pharmaceuticals, cosmetics, industrial- and agro-chemicals. This report describes the outcome of the discussions and recommendations (a) to reduce the number of animals used for determining the ADME properties of chemicals and (b) for considerations and actions regarding *in vitro* and *in silico* assays. These included: standardisation and promotion of *in vitro* assays so that they may become accepted by regulators; increased availability of industry *in vivo* kinetic data for a central database to increase the power of *in silico* predictions; expansion of the applicability domains of *in vitro* and *in silico* tools (which are not necessarily more applicable or even exclusive to one particular sector) and continued collaborations between regulators, academia and industry. A recommended immediate course of action was to establish an expert panel of users, developers and regulators to define the testing scope of models for different chemical classes. It was agreed by all participants that improvement and harmonization of alternative approaches is needed for all sectors and this will most effectively be achieved by stakeholders from different sectors sharing data.

© 2010 Elsevier Ltd. Open access under CC BY-NC-ND license.

## Contents

1. Introduction	591
2. Regulatory framework and views from regulators	591
2.1. Pharmaceuticals	591
2.2. Chemicals	591
2.3. Pesticides	593
2.4. Cosmetics	593
3. Use of <i>in vivo</i> and <i>in vitro</i> assays for risk assessment by different industry sectors	594
4. Information gained from <i>in vitro</i> assays	596
4.1. Absorption/exposure	596
4.2. Metabolic pathway identification and clearance	597
4.3. Selection of most relevant species	598
4.4. Drug–drug interactions in pharmaceutical safety evaluation	598
4.5. Mechanistic understanding	598
5. Recommendations from the workshop for <i>in vitro</i> assays	598
5.1. Apply considerations for <i>in vitro</i> assays (all sectors)	598
5.2. Standardize and promote <i>in vitro</i> assays (all sectors)	599
5.3. Avoidance of false positives (all sectors)	599
5.4. Mechanism-based toxicity assays should be further developed and validated (all sectors)	599
5.5. Actions related to <i>in vitro</i> assays (all sectors)	599
6. Information gained from <i>in silico</i> models	599
7. Recommendations from the workshop for <i>in silico</i> assays	600
7.1. More extensive use of QSAR by all industry sectors	600
7.2. Further development of PBBK models (all sectors)	600
8. Recommendations by the workshop for refining and reducing <i>in vivo</i> assays	600
8.1. Microdosing (pharmaceutical sector)	600
8.2. Incorporation of additional endpoints (chemical sector)	600
8.3. Issues in information sharing (all sectors)	600
9. Proposed actions from the ADME workshop to develop further alternative models and the 3R concept	600
9.1. Immediate actions	600
9.2. Additional actions	600
10. Conclusions	601
Conflict of interest	601
Acknowledgements	601
References	601

## 1. Introduction

Evaluation of the rates and extents of absorption, distribution, metabolism, and excretion (ADME) of compounds is a fundamental part of the in-depth understanding of the toxicological and pharmacological effects they may exert on humans and animals. Traditionally, ADME studies have been carried out using animals and, for certain industrial sectors, *in vivo* studies still have to be performed according to European regulatory frameworks. However, the development of non-animal test methods (i.e. “alternative” assays which may include *in silico* and *in vitro* models, as well as decision tree strategies to reduce animal testing) is strongly promoted within all industrial sectors in order to produce safety data that are more relevant to humans and to replace animal studies currently in use (Horizontal Legislation, 2008, agro-chemicals EU regulation: Council Directive 91/414 revision). The urgency for the cosmetic industry is more imminent since the use of certain *in vivo* animal studies (e.g. genotoxicity, eye and skin irritation and acute toxicity) has already been banned due to the 7th Amendment to the Cosmetics Directive and *in vivo* ADME studies will be banned in 2013.

*In vitro* biotransformation assays have been used routinely for decades but none have been validated for risk analysis (Blaauboer et al., 1994; Coecke et al., 1999). Nevertheless, the value of *in vitro* assays in assessment of chemicals is exemplified by their use in the drug candidate selection process in the pharmaceuticals industry which has proved quite successful in providing estimates of human bioavailability and clearance (Cai et al., 2006). The Organisation for Economic Co-operation and Development Technical Guideline (OECD) TG 428 describes an *in vitro* assay for testing dermal absorption but no similar detailed Guidelines exist with respect to the use of sub-cellular fractions, primary cells and *in silico* methods to predict metabolism (biotransformation). Nevertheless, tiered testing strategies for assessing metabolism have been suggested and reviewed previously (ECVAM, 2002; Coecke et al., 2005a). Models used to identify ADME properties (as well as organ-specific toxicities of chemicals) are summarised in Table 1, together with information regarding recommendations by the regulatory authorities and validation status. There is also a number of Quantitative Structure Activity Relationship (QSAR) models that are available to both industry and academia and these include but are not limited to the OECD toolbox (Table 2). Supporting activities from industry, European Commission and Academia to enable the development of non-animal models are summarised in Table 3.

An EPAA workshop was held in Duesseldorf on 24th/25th November, 2008, and was attended by scientific experts in the pharmaceutical, chemical, pesticide and cosmetic industries, by regulators, as well as by academia. Participants included representatives of the Scientific Committee on Consumer Safety (SCCS), European Centre for the Validation of Alternative Methods (ECVAM), European Food Safety Authority (EFSA) and Directorate General (DG) for Research. The aim of the workshop was to discuss how to implement *in vitro* ADME test systems as part of Integrated Testing Strategies (ITS) for the testing of cosmetics, pharmaceuticals and industrial chemicals and pesticides (including agro-chemicals such as herbicides, fungicides, or insecticides). The present report presents the outcome of the break-out group discussions in describing how *in vitro* assays may be used within different industry sectors and how regulators view *in vitro* data. It also outlines international projects aimed at developing alternative test models. In addition, the break-out sessions discussed the suitability of *in vitro* approaches to systemic toxicity and hazard identification for target organs and steps required to attain regulatory acceptance. Emphasis is placed on *in vitro* assays and their use in risk assessment issues including preliminary risk assessment such as for prioritisation and deprioritisation, rather than in targeted

risk assessment *per se*, since this is markedly different between industry sectors and is out of the scope of this paper.

## 2. Regulatory framework and views from regulators

The use of animal assays is different across industries, whereby *in vivo* studies are required in one sector but banned in another. An overview of these differences and the agencies which affect the use of animals is described below.

### 2.1. Pharmaceuticals

The European Medicines Agency (also known as the EMA) is a European agency which evaluates pharmaceuticals. In the USA, the equivalent agency is the Food and Drug Administration (FDA). Both the EMA and FDA evaluate and monitor products, as well as developing technical guidance (and guidance documents) and providing scientific advice to sponsors. According to EU Directives (EU Directive 65/65/EEC, 1965 and subsequent amendments), in order to bring a drug onto the market and before it has even been tested “first in man” its safety should be tested in animals – with the exception of certain genotoxicity tests (e.g. Ames assay). The Directive recommended that the use of animals should be limited for ethical and animal protection and welfare reasons and efforts should be made to develop new techniques which would produce the same quality of information as *in vivo* studies. It was for this reason that ECVAM was created in 1992, following a Communication from the Commission to the Council and the Parliament in October 1991. The requirement in Directive 86/609/EEC was to protect animals used for experimental and other scientific purposes and to actively support the development, validation and acceptance of methods which could reduce, refine or replace the use of laboratory animals. Therefore, although the pharmaceutical industry continues to develop new non-animal assays, this industry has not been pressured by regulators into switching from *in vivo* assays to *in vitro* alternatives to test drugs during the development process.

### 2.2. Chemicals

EU Chemicals Agency (ECHA) is the agency which manages the technical, scientific and administrative aspects of the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) regulation. The REACH regulation came into effect in June 2007 and was designed to regulate the manufacture, import, marketing and use of industrial chemicals (including ingredients used for formulations regulated otherwise such as pesticides and cosmetics). Manufacturers, importers and downstream users must demonstrate that the manufacture/import/use of a substance does not adversely affect human health and that risks are adequately controlled. This applies only to chemicals that are produced and/or imported in volumes of 1 tonne or more per year and it was expected to apply to tens of thousands of existing and new chemicals but over 143,000 chemical substances marketed in the European Union were pre-registered by the 1 December 2008 deadline ([http://echa.europa.eu/sief\\_en.asp](http://echa.europa.eu/sief_en.asp); Hartung and Rovida, 2009). The need for determining the toxicokinetics (TK) profile is listed in Annex 1 (Section 1.0.2) of the legislation but in Annexes (VII–X) it is not specifically required and its consideration is needed only if these data are available (Annex VIII–X). However, REACH does provide guidance (guidance on information requirements and chemical safety assessment, Chapters R.7C and R.8) on the use of TK for selection of dose, route of administration and test-species, as well as on route-to-route extrapolation in the derivation

**Table 1***In vitro* models used for identifying ADME and specific organ toxicity.

Endpoint	Organ	Model	Comments
<i>Absorption</i>			
Intestinal absorption, P-gp efflux	Intestine	Caco-2 cells (Hubatsch et al., 2007) MDCK (Irvine et al., 1999) PAMPA membrane, artificial lipid layers (Avdeef et al., 2005)	Validated for prediction of substrates or inhibitors of P-glycoprotein (Elsby et al., 2008) Absorption values may vary, depending on multiple factors (culture, passage etc.) New model validated as more predictive than PAMPA model (Chen et al., 2008)
Dermal absorption	Skin	Human skin static or flow-through diffusion cell models, using (partly) standardized <i>in vitro</i> conditions and human, rat or pig skin	OECD and EU guidelines are available (EU SANCO 222 and OECD TG 428, SCCS/1358/10)
Absorption across the BBB	Blood:brain barrier	Brain microvascular endothelial cells (Porcine, bovine), cell lines, artificial lipid layers (Garberg et al., 2005; Prieto et al., 2004)	No single model predictive, a battery of tests recommended (Garberg et al., 2005; Prieto et al., 2004)
<i>Metabolism</i>			
Metabolite profile	Liver	Microsomes, S9, hepatocyte suspensions and cultures	Ubiquitously used by industry. Their use for drug metabolism is reviewed by Bjornsson et al., 2003
	Skin	Human keratinocytes, HaCaT cells, <i>ex vivo</i> human skin	Used to determine presence of metabolites. The skin contains detoxification enzymes such as <i>N</i> -acetyl transferases (Goebel et al., 2009) but also expresses FMOs and CYPs (Janmohamed et al., 2001)
Clearance	Liver	Recombinant systems (human CYPs expressed in <i>E. coli</i> , baculovirus), hepatic sub-cellular fractions (S9 or liver microsomes), hepatocytes in primary cultures or suspension, liver slices	Recombinant CYPs suitable for “frontline” predictive human metabolism studies in early drug discovery (McGinnity and Riley, 2001). All liver-derived models provide useful information for prediction of metabolic routes, rates and interactions (Pelkonen et al., 2001)
Induction	Liver	Hepatocytes (e.g. human primary cultures) (Shou et al., 2008) Nuclear hormone receptor binding and activation (e.g. PXR) (Cui et al., 2008) Cell lines, e.g. LS180 (Harmsen et al., 2008)	FDA, 2006 guideline recommends either fresh or cryopreserved human hepatocytes for this assay (FDA, 2006)
Inhibition	Liver	Microsomes (from pools of >20 donors), hepatocyte suspensions and cultures, recombinant systems (e.g. human CYPs)	FDA recommends the use of microsomes but hepatocyte assays are in development (to study transporter effects and phase 2 metabolism) (FDA, 2006)
<i>Excretion</i>			
Excretion	Kidney, liver	Primary proximal tubule cell lines (Liang et al., 1999) Primary human hepatocyte sandwich cultures (Bi et al., 2006)	At present there is no model to reliably predict biliary or renal excretion
<i>Toxicity</i>			
Phototoxicity	Skin	NRU Balb 3T3 photocytotoxicity (Spielmann et al., 1998)	Validated since 2000, in annex V of directive 67/548; OECD 432 since 2002
Corrosivity	Skin	Trans-electrical resistance (TER) test (Archer and Liebisch, 1998) Human reconstructed skin models (Spielmann et al., 2007)	Validated since 2003; OECD TG 430
Skin irritation	Skin	<i>In vitro</i> membrane barrier test (Corrositex™ Membrane) EpiSkin™ and EpiDerm™ reconstructed skin models (Spielmann et al., 2007)	Validated since 2007; OECD TG 435. EpiSkin is expected to be endorsed as full replacement of the <i>in vivo</i> test OECD TG 431. EpiSkin™, Skinethic™ and EpiDerm™ are validated stand-alone replacements for the rabbit Draize test, distinguishing between skin irritating (R38) and non-irritating (no-label) chemicals (ESAC, 2008)
Skin Sensitisation	Skin	A combination of skin penetration, protein or peptide binding assays, activation of dendritic-like cells or cell lines	Sens-it-iv program initiated in 2005
Eye irritation	Eye	Bovine Corneal Opacity and Permeability (BCOP) (OECD 437); Isolated Chicken Eye test (OECD 438) assay	Partial replacements for the <i>in vivo</i> rabbit eye irritation test. Recommended by the OECD for use as part of a tiered testing strategy for classification and labelling within a specific applicability domain
Mutagenicity		<i>In vitro</i> test battery (Kirkland et al., 2005): Ames assay (OECD TG 471); chromosomal aberration (OECD TG 473); <i>in vitro</i> micronucleus test (OECD Draft TG 487); mouse lymphoma thymidine kinase	SCCP recommended a test battery for cosmetic ingredients (SCCP/1005/06)
Carcinogenicity		SHE (LeBoeuf et al., 1996) and Balb/c 3T3 (Maeshima et al., 2009) cell transformation assays	OECD TG 495
Reproductive toxicity		EST assay (ESAC, 2001; Bremer et al., 2002)	ReproTex program initiated in 2005
Neurotoxicity	Brain and CNS	Organotypic cultures derived from undifferentiated embryonic brain or spinal cord tissue (Braun et al., 2006) Re-aggregating brain cell culture (Braissant et al., 2002) Primary dissociated culture (Bal-Price and Brown, 2001) Immortalized human and rodent cell lines (Hong et al., 2003; Coecke et al., 2002)	Models which reflect specific but not all processes <i>in vivo</i> neural development. Based on mechanistic processes <i>in vivo</i> . Reviewed by Coecke et al., 2007
Acute Systemic Toxicity		Standard cytotoxicity endpoints, e.g. MTT, NRU using non-hepatic cell lines (e.g. 3T3), non-metabolizing cell type (e.g. V79) cells transfected with specific CYPs (Coecke et al., 2001), metabolic competent model (primary hepatocytes)	Can be used as part of decision-tree testing strategies for acute systemic toxicity and TK with respect to the requirements of the EU REACH legislation (Combes et al., 2008). ACuteTox program to replace <i>in vivo</i> acute toxicity



Table 1 (continued)

Endpoint	Organ	Model	Comments
Long-term toxicity		(Dierickx and Scheers, 2002; Clothier et al., 2006; Vignati et al., 2005), HPCT-1E3 model (Kneuer et al., 2007), Fa32 cells, HepG2 cells (Dierickx, 2000), genetically engineered NIH-3T3 or V79 cells (Bull et al., 2001)	testing with <i>in vitro</i> basal cytotoxicity models – initiated in 2005 (Clemenson et al., 2006)
		Nephrotoxicity cell lines: RPTEC-TERT1 (Wieser et al., 2008) and HK-2 (6th Framework STREP: Predictomics – Short-term <i>in vitro</i> assays for long-term toxicity (Jennings et al., 2009)	These cell lines are being used in Predict-IV

of a DNEL. Each chemical should be registered with ECHA, along with information on properties, uses and safe handling practices. If based on the current regulations, REACH will take many years and require vast numbers of animals (the latter number is under strong dispute and ranges between 9 million (ECHA, 2009) and 54 million (Hartung and Rovida, 2009)). Therefore, the REACH regulation challenges the chemicals industry to develop rapid, relevant, cost-effective *in vitro* assays to reliably predict human toxicity. In addition to drawbacks such as lack of regulatory acceptance another challenge for *in vitro* assays is that multiple models are needed to replace one *in vivo* model.

### 2.3. Pesticides

The European Food Safety Authority (EFSA) is a European agency whose role is to provide independent scientific advice and information in the form of opinions and technical reports to support Community legislation and policies and to collect and analyse data allowing assessment and monitoring of risks in the food and feed sector. The work of EFSA is mainly carried out in different expert panels dealing with, besides other food related fields, for instance with food additives, genetically modified organisms, food contaminants, transmissible animal diseases and pesticides and their residues. In a new regulation (EU, 2010), the EU Commission recommended that alternative models should include *in vitro* and *in silico* methods, as well as reduction and refinement of *in vivo* tests. Specifically for ADME determination, the EU Commission favoured the use of *in vitro* models from the same species as those used in pivotal studies and in human materials (microsomes and intact cell systems).

A risk assessment method considering the 3Rs currently explored by the EFSA is the Qualified Presumption of Safety (QPS) approach for micro-organisms. The QPS approach is based on the presumption that if for a taxonomic group of micro-organisms safety concerns can be excluded, any strain of this group can be considered as safe and that consequently further assessment (also employing animal tests) can be waived, thus reducing unnecessary animal tests.

In the European Union (EU) risk assessment and authorisation of plant protection products (PPPs) was at the time of the workshop carried out according to the provisions laid down in Council Directive 91/414/EEC (EFSA, 2007). This directive has been replaced by Regulation (EC) 1107/2009 of the European Parliament and the Council which will be fully applicable by 14th June 2011 (EU, 2010). PPPs that are designed to control pests are toxic by definition and are normally actively brought into the environment. Therefore, extensive testing before any decision on authorisation is mandatory. Testing requirements for the assessment of active substances with respect to possible human health effects include a battery of *in vivo* tests (acute, subchronic and chronic tests, reproduction toxicity) and are laid down in Annex II to Directive 91/414/EEC while in Annex III testing requirements for the final plant protection product are listed. The same data requirements are laid down also in the new regulation.

In an opinion that was formally adopted in 2007 (EU, 2007) the PPP and their residues (PPR) panel has provided recommendations for a revision of the data requirements of the directive and the new regulation, respectively. Several of these recommendations would reduce animal testing and animal use in the future. Recommendations given are for instance:

- Considering the application of PBBK modelling for assessing ADME.
- Waiving dermal acute tests when the oral toxicity and dermal absorption is low.
- Waiving *in vivo* genotoxicity tests when *in vitro* tests are negative.
- Employing the local lymph node assay for the assessment of skin sensitisation.
- Reviewing the questionable requirement of a 1-year dog study.
- Waiving, under certain circumstances, the conduct of a carcinogenicity study in mice.

Within the frame work of a new guidance document on the definition of pesticide residues for dietary risk assessment, the PPR Panel Unit is exploring on a large scale the applicability of alternative scientific tools not involving animal testing, like read-across and grouping of chemicals, QSAR and also the TTC approach for the assessment of the toxicity of pesticide metabolites that are present in food commodities.

### 2.4. Cosmetics

The Scientific Committee on Consumer Safety (SCCS) is an independent scientific committee (managed by the Directorate General for Health and Consumer Protection of the European Commission), which provides scientific advice to the Commission on non-food related issues. Cosmetics legislation is different from that of other sectors and is, across the EU, based on the Cosmetics Directive 76/768/EEC (EU, 1976). The 6th Amendment to the Directive (EU, 1993) requires that for each cosmetic product a safety dossier is available based upon the risk assessment of the individual ingredients (Pauwels and Rogiers, 2004) and not on that of the final product, as is the case in the USA. The 7th amendment (2004) prohibited the testing of finished cosmetic products in animals. Furthermore, a marketing ban on cosmetic ingredients tested *in vivo* for genetic toxicity, acute toxicity, eye irritation and skin irritation, came into effect on 11th March, 2009. The ban on reproductive toxicity, repeat dose toxicity and TK is expected to become effective in 2013. Whereas clear testing and marketing deadlines (11th March 2009 and 11th March 2013) are mentioned in the legislative texts, it is also clear that the scientific progress that would allow meeting these deadlines is not yet achieved. It is therefore urgent for the cosmetics industry to develop validated assays that fully replace animal studies for these endpoints in the future.

Although the SCCS welcomes the use of alternative methods once they have been validated, the Committee is confronted with the fact that still today the majority of the results present in the safety dossiers are based on animal studies. In particular, for active

**Table 2***In silico* models used for identifying specific organ toxicity and ADME properties.

Model	Comment
OECD QSAR Application Toolbox	Incorporates information into a logical workflow by grouping chemicals into chemical categories ( <a href="http://www.oecd.org">www.oecd.org</a> ). Evaluates the hazard based on the overall data set of the category, which must not have every chemical tested for every endpoint. Read-across from one tested chemical to an untested chemical is carried out to fill the data gaps
TOPKAT (Toxicity Prediction by Komputer Assisted Technology)	A statistically based system consisting of a number of robust, cross-validated QSAR models ( <a href="http://www.accelrys.com">www.accelrys.com</a> ) derived from large data sets of toxicological information from the literature. Chemicals are characterized according to structural, topologic, and electrotopologic indices. This system contains models based on data from 1258 compounds ( <a href="http://www.accelrys.com">www.accelrys.com</a> ) and can differentiate between irritants and non-irritants
DEREK (Deductive Estimate of Risk from Existing Knowledge)	A knowledge based system comprising a number of structural rules (based on strongly acidic and basic features which relate to the parent molecules) that aim to encode structure–toxicity information with an emphasis on mechanisms (Sanderson and Earnshaw, 1991)
DSS (Decision Support System)	The DSS SICRET (Skin Irritation Corrosion Rules Estimation Tool) model consists of a number of rules (known as the Gerner rules) based on physicochemical characteristics (such as melting point, logP and aqueous solubility) to exclude irritation, and structural alerts to include and predict corrosive chemicals (SICRET) and irritants (Saliner et al., 2007)
MEGen (Model Equation Generator)	This is a free web-based PBBK model equation generator and parameter database developed at the Health and Safety Laboratory as part of a joint industry project promoting the rapid generation of PBBK models ( <a href="http://xnet.hsl.gov.uk/megen">http://xnet.hsl.gov.uk/megen</a> )
ECOSAR	A library of QSARs which predicts aquatic toxicity and an expert system for selecting the appropriate QSAR
OncoLogic®	Cancer Expert System: Predicts concern levels for cancer potential based on “knowledge rules”
PBT Profiler	Estimates Persistence, Bioaccumulation and Toxicity and distribution in water, soil, sediment, and air using a Level III multi-media model
MultiCASE	The Multiple Computer Automated Structure Evaluation (MultiCASE) program uses a special type of algorithm to automatically identify chemical moieties that could lead to genotoxicity and deselect chemicals which lack structural alerts (e.g. high molecular weight chemicals such as polymers and chemicals which are only exposed dermally)
ADMEWorks	ADMEWorks can be used for predicting chemical and biological properties of compounds based on molecular structures (physicochemical, topological, geometrical, and electronic properties) and data on the property of interest (Hayashi et al., 2005)
Vitic Nexus database	A chemically intelligent database, which can recognise and search for similarities in chemical structures ( <a href="http://www.lhasalimited.org">www.lhasalimited.org</a> )

ingredients, a Margin of Safety (MoS, see Section 3) is calculated, based upon the lowest “no observed adverse effect level” (NOAEL), obtained either via a repeated dose toxicity test or a developmental toxicity study. Furthermore, the dermal absorption value and the calculated exposure level are also taken into consideration in the MoS calculation. Together with the results from skin/eye irritation tests, skin sensitisation assays and mutagenicity/genotoxicity screening batteries, the safety evaluation commonly is completed.

In the cosmetic field, specific additional studies are only performed with the purpose of elucidating mechanisms and/or to reduce TK or toxicodynamic underlying the minimal MoS value of 100. With the exception of the *in vitro* dermal absorption study, separate TK/biotransformation studies do not form key parts of current cosmetic dossiers. This, however, does not imply that the cosmetic sector would not be interested in the development of such alternatives – quite the opposite. One example is the development of sound xenobiotic biotransformation systems (e.g. appropriate functional cell lines) that could subsequently be used in an integrated approach next to repeated dose toxicity studies, developmental and/or mutagenicity/genotoxicity studies and possible alternative non-animal methods. Past experiences have shown that *in vitro* methods do not deliver reliable results and, together with a lack of a sound metabolic system, may constitute a major hurdle in the development of relevant *in vitro* assay systems.

In the cosmetic area, in addition, the availability of a good *in vitro* mutagenicity/genotoxicity battery is crucial. An in-depth study of 194 SCCP dossiers between 2002 and 2006 showed that the *in vitro* predictive potential alone is insufficient. Indeed, in that period 19 compounds were found positive *in vitro*, but negative in the confirmatory *in vivo* assays, meaning that these compounds would have been lost without the overriding animal testing possibility (Rogiers and Pauwels, 2008). With respect to skin sensitisation, an *in vitro* method that would predict the conversion of a pro-hapten into a hapten would be a significant improvement. Finally and importantly, it has repeatedly been acknowledged that examination of biotransformation and TK in general appear to be the ideal starting point for future long-term toxicity 3R-strategies.

### 3. Use of *in vivo* and *in vitro* assays for risk assessment by different industry sectors

Risk assessment in all sectors usually consists of hazard identification, dose–response assessment (together hazard characterization or effects assessment) and exposure assessment (which, together with effects assessment, forms the risk characterization) (Van Leeuwen, 2007). Animal data is used to extrapolate to humans and specifically to estimate the exposure level which would lead to a specific level of risk (for non-threshold effects) or a threshold below which no adverse effects are measurable (for threshold effects). A default combined safety factor in use for extrapolation of animal data to (sensitive) humans is 100 and has been used by FDA since the mid-50s (Lehman and Fitzhugh, 1954). It has since been adopted by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) and by the Joint FAO/WHO Expert on Pesticide Residues (JMPR) to define the Acceptable Daily Intake (ADI) (Truhaut, 1991). For other chemicals (at least in the EU) such as industrial chemicals and biocides, the MoS is calculated using two main scaling safety factors, namely, inter-species differences and intra-species differences (Renwick and Lazarus, 1998). It is used to extrapolate from a group of test animals to an average human, taking into account inter-species differences in kinetics and dynamics and variability in human kinetics and dynamics to account for sensitive subpopulations (Fig. 1).

Fig. 2 is a general scheme, generated as a result of break-out group discussions, on the use of alternative approaches used by different industrial sectors and how they are often used as compounds progress from identification to products, along the development pipeline. Naturally, there are a number of similar approaches where it is not only ethical to avoid animal testing but it makes good business sense to screen compounds for both efficacy and safety using appropriate non-animal models. The point at which animal tests come into the research and development process may be driven by regulation or by the lack of an alternative for the evaluation being undertaken. It should be

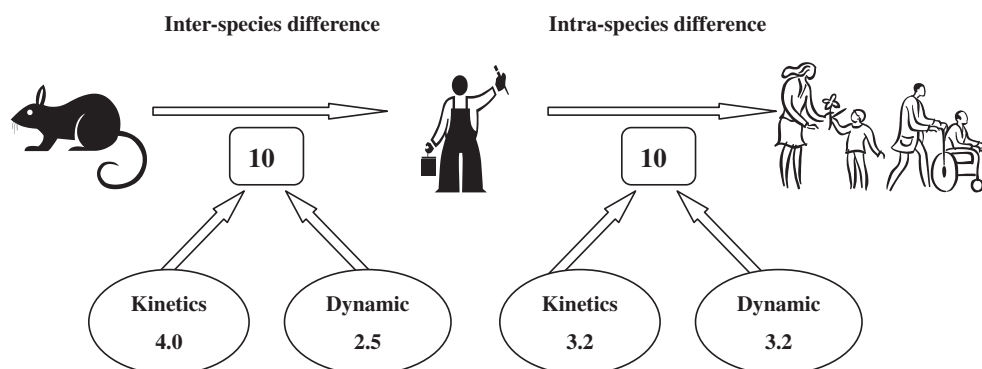
**Table 3**  
Supporting activities from industry, European Commission and Academia.

Activity	Description of activities
EPAA	Main objectives include mapping of past and current 3R activities to better inform the planning and prioritisation of subsequent actions; prioritisation, promotion and implementation of future research based on the application of the 3Rs; dissemination and implementation of best practice in the use of the 3Rs. EPAA has recognised the need for interaction between academia, industry and regulators to find ITS, or “building blocks”, which are reproducible, transferable and mechanistically predictive. Major challenges in validating alternative assays were discussed during an EPAA–ECVAM Workshop on ‘Overcoming barriers to validation of non-animal partial replacement methods/Integrated Testing Strategies’ in 2008 (Kinsner-Ovaskainen et al., 2009)
ECVAM	ECVAM works in co-operation with other organizations to develop standardized and validated methods for regulatory purposes. Regulators have to be confident in the alternative tests and, in order to achieve this, <i>in vitro</i> toxicity studies should be carried out according to Good Laboratory Practice (GLP). The ECVAM Workshop Report concluded that the OECD documentation on GLP should be applied as far as possible (Cooper-Hannan et al., 1999). This led to the publication of an OECD Advisory Document in 2004, which outlined the “Application of the Principles of GLP to <i>in vitro</i> Studies” (OECD, 2004). As an aid to the production of robust data, an ECVAM Task Force Report provided guidance on good cell culture practice (GCCP) (Coecke et al., 2005b). ECVAM has focussed on the use of human models
ACuteTox	This project is an EU-funded project (6th Framework Programme (FP6)) focussing on the development and pre-validation of a simple and robust <i>in vitro</i> testing strategy for prediction of human acute systemic toxicity. The aim is to replace animal acute toxicity tests currently used for regulatory purposes by <i>in vitro</i> tests which allow for detection of bioactivated toxins. The project uses the paradigm that acute toxicity of a compound is determined by its basal cytotoxicity. However, this may not hold for many compounds for which acute toxicity is due to specific toxicity, rather than basal toxicity. This has been addressed in Work Package 6.1 in which the cytotoxicity reference chemicals, as well as 5 additional compounds which require bioactivation, have been compared primary hepatocytes (metabolically competent) and HepG2 cells (relatively poor metabolic competence). Compounds which are more toxic to hepatocytes suggest that they require bioactivation to elicit cytotoxicity. In Work Package 7 (investigating neurotoxicology, nephrotoxicity and hepatotoxicity), different measurements of cytotoxicity were compared with the MTT assay. Following statistical analysis and data mining, the best combinations of <i>in vitro</i> tests that give a relatively good correlation with <i>in vivo</i> (rat and human) data were identified. Thirty-three compounds will be tested in the selected assays under blind conditions and the results will be used retrospectively to validate the preliminary testing strategy
Predict-IV	This project sets out to profile the toxicity of new drugs using a non-animal-based approach integrating toxicodynamics and biokinetics. The aims include the development of new strategies for drug safety assessment focusing on non-animal test systems. Other goals are to deduce MoSs and identify early biomarkers in human hepatotoxicity, nephrotoxicity and CNS toxicity. Long-term human models for non-target organ specific toxicity and bioactivation systems and co-culture models for specific toxicity are also being evaluated. The aim is to analyse samples, using genomic, transcriptomic and metabolomic profiling, from each model which have been treated with the same compound and compare outcomes from each endpoint. The compounds selected are those with well-described toxicities and kinetics in animals and partly also in humans
OSIRIS	OSIRIS (Optimised Strategies for Risk Assessment of Industrial Chemicals through Integration of Non-Test and Test Information) aims to develop an ITS to enable a significant increase in the use of non-testing information for regulatory purposes. One of the so-called “interlinked Research Pillars” focuses on exposure-informed testing (triggering and waiving). The reduction of the use of animals could be implemented in a number of ways and some of these issues have been discussed previously (Barton et al., 2006; Creton et al., 2009). Results from recent studies are shown in the OSIRIS web site <a href="http://www.osiris.ufz.de/index.php?en=18585">http://www.osiris.ufz.de/index.php?en=18585</a>
START-UP 3Rs	“START-UP” is a coordination and support action coordinated by Prof. Vera Rogiers. A number of small 3Rs projects are supported which aim to establish a better link between academia and industry. The latest progress report is available at <a href="ftp://ftp.cordis.europa.eu/pub/fp7/docs/alternative-testing-progress-report-2009_en.pdf">ftp://ftp.cordis.europa.eu/pub/fp7/docs/alternative-testing-progress-report-2009_en.pdf</a>
EU Directorate General (DG) for Research	As a result of the high priority of developing alternatives to existing repeated dose systemic toxicity testing, the EU Directorate General (DG) for Research and Colipa to formulate a specific research strategy to address this problem. Two of its main objectives are to (1) encourage collaboration between industry and academia to gain leadership in key technology areas and (2) promote ideas by supporting basic research at the scientific frontiers (implemented by the European Research Council). There are several FP6 projects still running in DG RTD which apply <i>in vitro</i> and <i>in silico</i> methods for human safety assessment. FP7 includes a significant number of new initiatives ranging from ITS to coordination to optimization of the use of the limited financial resources. More information on the on individual research projects and support actions can be found in the 2009 Progress Report on Alternative Testing Strategies <a href="http://cordis.europa.eu/documents/documentlibrary/106691831EN6.pdf">http://cordis.europa.eu/documents/documentlibrary/106691831EN6.pdf</a>

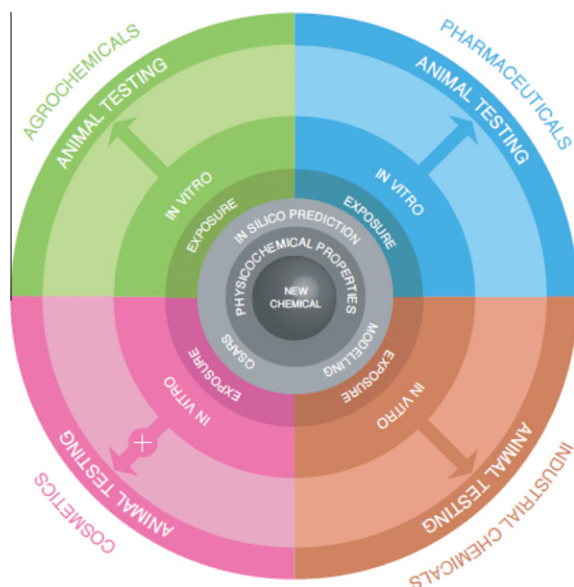
pointed out that strategies that involve a small number of animals at early stages of development may actually reduce the overall numbers of animal procedures that may have identified a toxicological issue much later in development. Therefore refinement

and reduction are often forgotten but still very important steps in the overall 3R target.

In all sectors an initial evaluation of new chemicals is often made based on their physicochemical properties, e.g. solubility,



**Fig. 1.** Uncertainty factors incorporated into the MoS used in risk assessment.



**Fig. 2.** Target diagram on the use of alternative approaches in the ADME safety testing area by different industrial sectors. The target for each sector is to undertake more extensive *in silico* and *in vitro* evaluation of new chemical entities in early development so that fewer animal studies are needed as the development process moves towards animal testing that is often a regulatory requirement. Although all sectors actively pursue alternative approaches for ethical and practical reasons, the cosmetics industry is actually prohibited from using animals for specific toxicity endpoints. (Diagram prepared by Jon Heylings).

logKow P, pH, pKa and molecular weight. Assumptions as to likely corrosive effects can be made if the chemical has an 'extreme' pH value ( $\geq 11.5$  or  $\leq 2$ ), especially if it is to be applied topically (it may be corrosive or a skin or eye irritant, for example). In order to screen potentially thousands of compounds, many companies incorporate the use of *in silico* models (listed in Table 2).

As part of a risk assessment of possible systemic toxicity, in addition to the characterization of the hazard, the likely systemic exposure of the chemical has also to be taken into account. This will differ between industries since pharmaceutical companies usually aim to reach a significant target therapeutic plasma concentration and assess the compound on a risk-to-benefit basis. Since the intended exposure target is potentially high, consideration of the risk-to-benefit is important in the pharmaceutical industry (more so than the chemical or cosmetics industry) and the weight of this ratio may also differ according to the different product types (e.g. cancer therapy versus diabetes). For chemicals industries it is key to assess the likely exposure under occupational conditions.

*In vitro* assays are used by all sectors of industry for safety testing but the need for *in vitro* models in risk assessment will differ according to the needs of the different industrial sectors and the specific question that needs to be addressed. Appropriate models of varying complexity are often used by different sectors to address specific organ toxicity. For example, there are a number of models used for investigating liver metabolism and toxicity which have certain characteristics as well as limitations in their use (summarised in Table 4). *In vitro* data may be more suitable for in-house decision-making within an industry sector, whereas the regulatory agency may ask for much more specific information on an effect seen *in vitro* (e.g. whether a specific transporter is involved in the clearance of a compound). Exposure-based waiving can be used as in-house method if, e.g. an *in vitro* assay shows that a target organ would not be exposed to a test compound, in which case, an *in vivo* study would not be needed.

In the pharmaceutical industry, animal studies have to be carried out for licensing of a medicinal product containing a new active substance but *in vitro* assays are used for screening, drug

candidate selection and drug–drug interaction information for Phase 1 clinical trials. ADME studies here are not necessarily conducted according to regulatory legislation. Moreover, studies which investigate the use of potential drug candidates can be performed under non-GLP conditions, especially for non-standard screening technologies, safety studies performed to support regulatory requirements (e.g. Investigational New Drug (IND) applications) should, in general, be GLP compliant. However, *in vitro* assays performed to predict toxicity may be carried out according to the FDA draft guidelines (FDA, 2006). These assays are included in Table 1. The pharmaceutical industry and, on a less routine basis, the chemical industry employ PBBK models to identify and reduce uncertainties in risk assessment (MacGregor et al., 2001; Delic et al., 2000). In terms of risk management, it should be kept in mind what constitutes an acceptable risk, depending on the industry and the purpose of the compounds under development.

Once an assessment of the source and likely exposure of a chemical is addressed, the risk can be characterized as an estimation of the incidence and severity of any adverse effects likely to result from actual or predicted exposure. For REACH chemicals, the level of exposure above which humans should not be exposed should be estimated, i.e. the DNEL (Derived No Effect Level). In the risk characterization, the exposure of each human population known to be, or likely to be exposed, is compared with the DNEL. The risk to humans can be considered to be adequately controlled if the estimated exposure levels do not exceed the DNEL. Calculation of the DNEL (Human Limit Value) involves a number of considerations such as uncertainty, extrapolation or assessment factors (inter-species, intra-species, exposure duration, route-to-route etc.) and should not be confused with the NOAEL (usually derived in animals). For agro-chemicals, *in vitro* assays can be used to compare metabolites produced by mammalian cells with those produced by plants and determine whether the toxicological evaluation of each agro-chemical sufficiently encompasses any crop residues of concern.

The acceptance and validation of *in vitro* models is most pressing for the cosmetics industry. They must consider the route and extent of exposure, since the skin is the main site of application of cosmetics, as a result, major focus has been placed on dermal absorption for which accepted *in vitro* methods are available. Other dermal models include human reconstructed skin models for use in genotoxicity testing (Munn et al., 2009). For other endpoints such as skin and eye irritation, scientifically accepted methods used in combination are being used as alternatives to animal models. In contrast to other sectors, the cosmetics industry is required by law to replace a number of *in vivo* animal tests with scientifically valid alternative approaches.

#### 4. Information gained from *in vitro* assays

In an optimal situation, ADME/TK are cross-cutting issues that are taken into account in all these processes, albeit not literally or specifically required in various sector legislations. To this end, scientifically justifiable – but not legally required – information may come from *in vivo* as well as *in vitro* assays which can be used by one or more sectors to determine ADME properties as well as understanding mechanisms of action. Examples of information gained from *in vitro* models are described below and listed in Table 1. A major challenge is that *in vitro* methods are needed that allow for a quantitative assessment of effects *in vivo*.

##### 4.1. Absorption/exposure

Safety assessors from all industry sectors will need to evaluate the exposure of a chemical to human health, whether it is intestinal absorption from an orally dosed drug, systemic exposure from



**Table 4***In vitro* liver models and their characteristics and limitations.

Model	Characteristic	Advantage	Limitation
Isolated perfused liver	<i>Ex vivo</i> organ culture	Complete liver enzymes and cofactors Liver architecture retained All liver cell types present Bile flow	Fresh tissue needed Short-term viability (2–3 h) Study of one or a few compounds only Humans excluded Low reduction of animal numbers
	<b>Closest to <i>in vivo</i></b>		
Liver slices	<i>Ex vivo</i> tissue culture	Complete liver enzymes and cofactors Most liver cell types present Cell–cell interactions	Fresh tissue needed Limited viability (~24 h) Damaged cells at the cut surface Marginal aeration of inner cells Slow transport Bile collection excluded Cryopreservation limited
	<b>Close to <i>in vivo</i></b>		
Hepatocytes	<i>Ex vivo</i> cell culture from livers or biopsies	Complete liver enzymes and cofactors Cryopreservation possible Higher throughput screening possible	Specific techniques and well-established procedures needed Liver architecture lost Enzyme levels decrease during culture Batch variability (e.g. viability, attachment efficiency)
	<b>Cell culture</b>		
Liver cell lines	<i>In vitro</i> cell culture	Good inter-experimental reproducibility Cryopreservation possible Suitable for high-throughput screening	Poor or absent enzyme expression Limited enzyme activities Genotype and phenotype instability Not many adequately characterized cell lines
	<b>Cell culture</b>		
Sub-cellular fractions	Organelle fraction suspension; endoplasmic reticulum-bound and cytosolic enzymes	Easy to use Suitable for high-throughput screening Cryopreservation possible	Microsomes contain only CYPs, FMOs and UGTs Require cofactor supplementation Extrapolation to <i>in vivo</i> limited Induction effects excluded Partial metabolic profile Short-term incubation
	<b>Enzyme preparation</b>		
Genetically engineered cells	<i>In vitro</i> cell culture or protein suspension; enzymes produced in endoplasmic reticulum of host cells (bacteria, yeast, mammalian cell lines or baculovirus); with or without cofactors	Good inter-experimental reproducibility Cryopreservation possible Suitable for high-throughput screening	Limited enzyme number per experiment Pilot study needed Short-term study Extrapolation to the <i>in vivo</i> situation limited
	<b>Artificial system</b>		

Modified according to Coecke et al. (2006), Guillouzo (1998), Plant (2004) and Pelkonen et al. (2004).

a dermally applied cosmetic or accidental exposure from a pesticide. Whereas the pharmaceutical industry is aiming to have good systemic exposure (high bioavailability), the chemical, pesticide and cosmetics sectors are likely to develop chemicals which are poorly absorbed. A number of cell lines, such as Caco-2, are routinely used to determine intestinal absorption. When used as part of a simulation model that takes into account solubility and dissolution in the gastrointestinal tract as well, they give a good prediction as to the extent of absorption (Thomas et al., 2008). Likewise, cell lines have been employed to predict penetration across the blood brain barrier, although these models still require some further development. The most relevant route of exposure for cosmetics, industrial chemicals and pesticides is the skin (although exposure via inhalation and the oral route can be very relevant as well), for which static or flow-through diffusions cell models have been standardized (as least in part) for use with human, pig and rat skin in OECD and EU context (OECD TG 428, (SANCO, 2004)). Moreover, there is on-going revision of the current guidance document on dermal absorption (SANCO, 2004).

#### 4.2. Metabolic pathway identification and clearance

The liver is the main site of metabolism of xenobiotics and therefore the majority of *in vitro* models to determine metabolic pathways and clearance are hepatic (hepatocytes, S9, microsomes

etc., Tables 1 and 4). For the potential occupational exposure of chemicals via the dermal route, metabolism in the skin is of importance since it has been shown to possess a number of drug metabolizing enzymes (Oesch et al., 2007). *In vitro* models used to evaluate skin metabolism include normal keratinocytes, cell lines such as HaCaT cells and *ex vivo* human skin (Table 1). For the pharmaceutical industry, knowledge of the enzymes involved in the metabolism of a compound can provide information of the likelihood of drug–drug interactions, possible problems due to polymorphic enzymes, disease, gender and age; and potential reactive metabolites. So-called “phenotyping” information can be used to provide individualized health care and stratified clinical trials. For cosmetics, human liver microsomes have been used to screen hair dyes for their potential to form reactive intermediates rather than carrying out *in vivo* assays which are also more labour intensive and expensive (Skare et al., 2009).

Many researchers focus on the cytochrome P450s (CYPs) since these are the major phase 1 enzymes responsible for the metabolism of the majority of pharmaceuticals on the market (Zuber et al., 2002). However, there are other non-CYP enzymes which may also metabolize compounds, such as the phase 1 alcohol dehydrogenases (Kollock et al., 2008)) and the phase 2 enzymes, sulfotransferases (SULTs), UDPGA-glucuronosyltransferases (UGTs) and glutathione S-transferases (GSTs) (Evans and Relling, 1999). It is important to include phase 2 enzymes such as GSTs in

metabolic studies to more completely reflect the physiological situation. In many cases phase 2 enzymes can detoxify substances and/or their phase 1 metabolites (e.g. paracetamol toxicity (Schnackenberg et al., 2008)). Identification of the enzyme(s) involved in the metabolism of a compound and understanding how metabolism may vary across and within species and across human subpopulations, e.g. poor metabolizers versus extensive metabolizers (Bogni et al., 2005), is very important for risk assessment (choice of test-species and possible use of a larger intra-species extrapolation factor). Another important use of *in vitro* metabolic studies is the use of these data to confirm the MoS (see Section 3). The use of a general 3.2 kinetic factor reflecting inter-individual variation may not cover metabolism by poor metabolizers or extremes of ages (Renwick and Lazarus, 1998; Dorne et al., 2002, 2003; Dorne and Renwick, 2005); therefore, the kinetic factor can be confirmed or adjusted according to the metabolic phenotype.

#### 4.3. Selection of most relevant species

Traditionally, the evaluation of species differences in metabolite formation has not been considered on a routine basis, mainly due to the uncertainty of the contribution of metabolites to the toxic effect. However, it is now evident that species differences in drug metabolizing enzymes can influence the toxicity of a compound across species (Uehara et al., 2008). Therefore, there is a need to carry out TK studies on rodent and non-rodent species in order to decide on the most relevant test-species for possible human effects testing. For example, according to the FDA guidelines (FDA, 2005), if a metabolite represents more than 10% of parent compound in human (defined as a major metabolite), then it should be present in the animal species tested. This emphasises the importance of establishing major metabolites produced by different species using *in vitro* assays so that they can be covered in animal toxicity studies. This line of guidance is also recommended by the EU Commission (EU, 2010). Following on from this, in order to evaluate non-clinical animal toxicology studies, the systemic exposure of the drug (quality, i.e. parent and/or metabolites, as well as quantity, i.e. extent and/or rates of formation) should be considered and compared between the test-species and humans (i.e. species-specific metabolism). This comparison is reasonable if the metabolic pathways are similar, however, in rare cases, if *in vitro* assays suggest that major metabolites produced in humans are not evident in animals, then further investigations into the toxicity of the metabolite are necessary. If it can be established that at least one animal test-species produces major metabolite(s) observed in humans, it can be assumed that the metabolite's contribution to the overall toxicity assessment has been taken into account. The use of *in vitro* assays, especially in early compound development, allows for selection of compounds and, when possible, the most suitable pre-clinical species, as well as flagging up compounds that may require additional toxicity studies to evaluate the contribution of the metabolites to the toxic effects (Coecke et al., 2005b).

#### 4.4. Drug–drug interactions in pharmaceutical safety evaluation

Drug–drug interactions are most relevant to the pharmaceutical industry since often more than one drug is purposefully given at therapeutic doses to treat multiple symptoms/causes of illness (i.e. polypharmacy). Unfortunately, one drug may alter the pharmacokinetics of the co-therapy drug and result in either the loss of efficacy or increased toxicity of the latter. Metabolic inhibition of drugs can be predicted using human liver microsomes whereas human hepatocytes are considered to be the “Gold Standard” for predicting metabolic induction (Table 1). Knowledge of potential

drug–drug interactions is a vital part of the candidate (de)selection process as well as aiding in the design of clinical interaction trials.

#### 4.5. Mechanistic understanding

Significant progress has been made in the understanding of cellular-response networks, i.e. a network of pathways involving a complex biochemical interaction of genes, proteins, and small molecules that maintain normal cellular function. Advances in our knowledge of the pathways are allowing researchers to investigate how they are altered by environmental agents and ultimately lead to toxicity. The US National Research Council (NRC) report in 2007 called for more human-cell-based, high-throughput assays that encompass a multitude of toxicity pathways and in response, NIH (National Institutes of Health) and EPA have entered into a formal collaboration known as Tox21 (Tox21, 2008). Their aims are to identify mechanisms of chemically-induced biological activity, prioritize chemicals for more extensive toxicological evaluation, and develop more predictive non-animal based models of *in vivo* biological response. Hopefully, this research will lead to toxicity models that are more scientific and cost-effective as well as models for risk assessment that are more mechanistically-based. Despite the advances, the resulting mechanism-based assays need validation or at least profound scientific evaluation before they can be used routinely. Often, the appropriate prediction evaluation occurs in parallel with assay development and ultimately leads to the streamlining of the assay. Parameters such as stability of solutions, proteins or even cell lines should be checked and standardized. Incubation times, storage, robustness (replicates for statistical analysis) are also some of many considerations companies make when validating assays (McGee, 2006).

### 5. Recommendations from the workshop for *in vitro* assays

The main priority for all industry sectors is the safety of the products and thus for people, animals and the environment and doing this with a reasonable the number of animals used and, in the case of the cosmetics industry, to replace *in vivo* assays entirely. Some of the priorities were discussed in break-out groups (each containing representatives from academia and industry and in some, representatives from regulatory bodies) from the workshop and are listed below. The sector(s) to which the priority applies most is shown in brackets. Topics that were discussed were not necessarily the views of all those who participated.

#### 5.1. Apply considerations for *in vitro* assays (all sectors)

Through discussions in the workshop, it was concluded that in order to interpret *in vitro* data, a number of considerations need to be made which include:

- Are *in vivo* and *in vitro* concentrations the same and is the *in vitro* concentration relevant to *in vivo*?
- Is the effective concentration the same as the concentration added to the *in vitro* system? Is the effective concentration *in vitro* translated correctly to the *in vivo* concentration?
- What are the volatility and binding properties (to plastic surfaces and/or to microsomal/cellular proteins) of the compound? This in turn affects the concentration to which the cells are exposed. In order to determine the free fraction of compound, solid phase micro extraction can be used (Pawliszyn, 1995; Vaes et al., 1996).
- What is the free concentration of test compound in incubation medium containing different concentrations of serum? The presence of serum can affect the free fraction of a compound as much as reducing it to 1000th of the nominal concentration added.

- Consideration of the contribution of multiple organs: *In vitro* assays focus on one organ, rather than the animal/human as a whole. Therefore, influences of other organs are not incorporated into the model and may cause an under- or over-prediction of a toxic effect. Section 4.1 describes how at least the **distribution into multiple organs** can be predicted using (physiologically-based biokinetic) PBBK modelling approaches.
- Consideration of the phenotype: A **pre-screening characterization of cell lines**, especially if they lack specific bioactivating/detoxification enzymes, should be carried out in order to interpret resulting data. A common understanding of HepG2 cells is that they have a low metabolizing capacity; however, there are reports of these cells having comparable enzyme activities to primary human hepatocytes (Westerink and Schoonen, 2007a,b) and such activities may be due to the source (and therefore sub-clones) and the type of basal medium used to culture and passage them (which have been shown result in different phase 1 and 2 enzyme activities of HepG2 cells (Hewitt and Hewitt, 2004)).
- Consider uncommon effects: Predictive models are based on certain assumptions and known common chemical and physiological processes and, as a result, may miss certain effects. For example, prediction of accumulation of test compounds in bone using *in vitro* assays has not been described so far.

## 5.2. Standardize and promote *in vitro* assays (all sectors)

There are many variables in metabolism assays which may affect their outcome; therefore, harmonization of these assays is needed. The harmonization of toxicity tests according to OECD guidelines began in the early 1980s. In addition the testing of the safety and efficacy of drugs is harmonized by the International Conference on Harmonisation (ICH). This has led to the effect of not just standardizing tests but reducing the number of animals used, since regulatory agencies around the world now accept the results of a test conducted according to such guidelines. Nevertheless, researchers have to work hard to convince regulators and the scientific community that some *in vitro/in silico* methods are sufficiently reliable to be used, albeit not yet for systemic toxicity endpoints. It was felt that stronger involvement of regulators in the early stages of the process should be encouraged so that it can be clarified through dialogue what information is needed for an assay to be validated. Additionally, the perception, or weight, of the information from *in vitro* assays should be correctly assessed and communicated between the researchers and regulators.

## 5.3. Avoidance of false positives (all sectors)

Care must be taken not to be “overly-efficient”! For one company, due to efficient in-house de-selection of test compounds, there were no positive genotoxic compounds in *in vivo* studies. Since there are false positive results from single and combined *in vitro* genotoxicity assays, de-selection of all positive responses in these assays may prevent the development of promising non-genotoxic compounds. Negative outcomes in *in vitro* genotoxicity assays (which exhibit high sensitivities) are accepted by regulatory agencies; however, this is not the case for other endpoints such as skin irritation. One Colipa (European Cosmetic Toiletry and Perfumery Association) project in progress is to refine current assays to avoid generation of false positives (project entitled “Reduction in the “false positive” rate of *in vitro* mammalian cell genotoxicity assays”, co-sponsored by Colipa, ECVAM and UK NC3Rs); likewise, the FDA is striving for highly predictive systems to avoid false positives.

## 5.4. Mechanism-based toxicity assays should be further developed and validated (all sectors)

Known toxic and adverse effects should also be defined for the kidney, heart, lung, CNS, immune system, adrenal and thyroid glands (endocrine disruptors). Information on known substances developed by the pharmaceutical and, if possible, other industries should be collected. This will help develop QSAR models and new assays (including active transport, signalling).

## 5.5. Actions related to *in vitro* assays (all sectors)

Workshop participants suggested two actions which may **aid the interpretation of data generated from *in vitro* assays**, such as:

- Integration of information from different models: Integration of data from separate organ *in vitro* assays may provide a better overview of toxicity. For example, the contribution of gut bacteria may be incorporated into an absorption model to allow the prediction whether a compound is (re)absorbed from the intestine as parent or metabolite followed by possible further metabolism by another organ.
- Evaluation of assays: **Performance assessment and evaluation of *in vitro* assays** is needed with appropriate controls (e.g. positive, negative and reference compounds) to confirm that the assay is functioning. For example, a cytotoxicity assay should include a positive compound which causes significant cytotoxicity (up to 100%) so that the assay can be shown to be sensitive. Likewise, an enzyme induction study should include a vehicle and positive control for the enzyme (e.g. CYP or phase 2 enzymes) under investigation to compare the potency of the induction effect of a test chemical and assess the dynamic range of the induction capacity of the test system. Contract Research Organisations usually do not validate *in vitro* assays that are not requested on a routine basis. A number of specialised assays are routinely used and these are often optimized in-house. Lastly, the perception of “validation” maybe different between companies and regulatory bodies.

## 6. Information gained from *in silico* models

A number of QSAR models exist (shown in Table 2) which can be used to prioritize chemicals and compare large numbers of chemicals using standardized criteria. Other mathematical models based on ADME properties are referred to as physiologically-based biokinetic (PBBK) models and are synonymous with physiologically-based pharmacokinetic (PBPK) models and physiologically-based TK (PBTK) models. The prediction of *in vivo* PK parameters such absorption, first pass effects and metabolism has been successfully demonstrated using the SimCyp PBPK model, which is a population-based simulator using physicochemical, *in vitro* and *in silico* data ([www.simcyp.com](http://www.simcyp.com)). In addition to PK prediction models, mathematical ADME models have been developed to assess TK properties (the effect of the chemical on the body) to address the 3R agenda (Bouvier d'Yvoire et al., 2007). The most predictive are those which are capable of integrating known physicochemical and chemical-specific *in vivo*, *in vitro* as well as *in silico* (QSAR) data (Blaauboer, 2003). A new paradigm is that toxicity is determined by the critical concentration and time of exposure to the critical compound (or metabolite) at the critical site of action of the compound. Biokinetics is an important part of this paradigm. PBBK models take into account the fact that organs are linked together. Knowledge of *in vitro* kinetics can be combined with *in vitro* toxicodynamic data and incorporated into a model to predict *in vivo*

systemic toxicity. An example of this is acrylamide for which *in vitro* data on neuronal toxicity was known (DeJongh et al., 1999).

To date, ADME software packages, although showing promising predictive capacities, especially for absorption and distribution, have not yet been sufficiently validated and still require improvements. A report of an expert meeting organized by COST B15 that reviewed the use of QSAR in drug screening (Boobis et al., 2002) suggested that predictions using QSAR are no worse than those made using *in vitro* tests, and have the added advantage that they need significantly less investment in technology, resources and time. The report went on to describe a lack of confidence in these approaches and that more effort should be made by the software producers towards more transparency, in order to improve the confidence of their consumers. It was also felt that controlled access to data from pharmaceutical companies would help to validate the models.

## 7. Recommendations from the workshop for *in silico* assays

### 7.1. More extensive use of QSAR by all industry sectors

If QSAR is used as the first step in risk assessment, then compounds that are flagged up as toxic can be de-selected, thus providing a 3Rs and cost-effective screening process. The workshop recommended that the basic parameters of the chemical should be considered (e.g. physicochemical properties) as well as its partitioning into the tissues (indicated by the octanol:water partition coefficient versus the fat:blood partition coefficient) and the physiology of the organ (e.g. structure, blood flow, metabolic capacity, etc.). In addition, there should be more data generated to add to the predictive power of models.

### 7.2. Further development of PBBK models (all sectors)

Further developments should combine *in vitro* and *in silico* data to feed PBBK models. To this end, increased efforts are needed to develop medium throughput systems to establish absorption (e.g. Caco-2), partitioning coefficients and metabolic parameters for the most important metabolizing organs, i.e. liver and skin. The use of publicly available tools such as the Model Equation GENERator (MEGen, <http://xnet.hsl.gov.uk/megen>, see Table 2) should be encouraged. Resulting PBBK models can be used to prioritize *in vitro* development projects. In order for a prediction model to be built, the extrapolation between the concentration of a compound in the incubation medium *in vitro* and the equipotent plasma concentration is a crucial step, involving predictive TK modelling. In this modelling approach, the metabolism of the compound should be taken into account, especially if it is rapidly metabolized and the nominal *in vitro* concentration is not constant. Drawbacks of *in vitro* models are that they have been developed mainly for screening purposes by the pharmaceutical industry and are not validated for certain categories of industrial chemicals. Therefore, training with the latter compounds and taking into account uncertainty is needed.

## 8. Recommendations by the workshop for refining and reducing *in vivo* assays

### 8.1. Microdosing (pharmaceutical sector)

This methodology allows for the determination of human pharmacokinetics of test compounds administered at doses much lower than the expected pharmacologically effective or toxic levels (FDA, 2008). Microdosing has been used as part of human drug clinical testing to evaluate drug ADME (Coecke et al., 2005b) but has not been widely accepted for testing chemicals. This is not used uni-

versally and is done on a case-by-case basis. This technology, once installed is cost-effective to study new chemical entities and has the advantage of requiring only very low doses of radiolabelled compounds. One limitation to this technology is that the dose has to be lower than 100 µg, thus if this is significantly different from the therapeutic dose and the pharmacokinetics profile is different, then the low dose pharmacokinetics data may have decreased relevance compared to the toxic/effective concentration. Another disadvantage of this method is that humans are purposely exposed to radiation for biomedical research and its use should therefore be justified (as recommended by the International Commission of Radiation Protection in Publication 62 (ICRP, 1991)). There are radiation dose constraints for volunteers under different conditions and these are discussed in the recommendations from the ICRP (ICRP, 2007).

### 8.2. Incorporation of additional endpoints (chemical sector)

In order to refine and improve existing *in vivo* study types, as well as reduce the number of animals used, for chemical testing, it was recommended to increase information gained from one study by incorporating additional endpoints into the study, e.g. using peripheral blood for metabolomics and the micronucleus (MN) test. It is noted that inclusion of more endpoints, e.g. kinetics, may be difficult to implement for small animals, e.g. mice. In addition, inclusion of positive controls for each endpoint may mean extra animals are needed, although, for some endpoints which have sufficient historical data, such as the *in vivo* MN test, additional positive controls are not an absolute requirement.

### 8.3. Issues in information sharing (all sectors)

The different industry sectors have generated a vast amount of data using similar models; however, the sharing of this data across sectors has not been as fast flowing. The workshop recommended the sharing of *in vivo* data, coordination and information exchange between research projects and sectors. Companies should be encouraged to share in-house additional data from long-term studies so that *in vivo* studies are not unnecessarily duplicated and *in silico/in vitro* methods can be validated.

## 9. Proposed actions from the ADME workshop to develop further alternative models and the 3R concept

### 9.1. Immediate actions

- Assess promising ADME/PBBK approaches which (a) have been entered into the EPAA in-house methods database or directly via member companies (internal) and (b) are available from false positive projects or others (external).
- Establish an expert panel, including users, developers and regulators, that defines the testing scope for the potentials and limitations of the models with a set of chemicals.
- Organize a series of follow-ups (with EPAA members, regulators and external stakeholders) to discuss the results and identify gaps and recommend ways forward.
- To engage the help of ADME experts from the member companies.

### 9.2. Additional actions

- Expert follow-up meeting: Review of developments and changes in the last three years with a focus on replacement/cosmetics (Eskes and Zuang, 2005). Participants should include the previous ECVAM panel, the EPAA workshop participants and selected participants from other sectors.



- ECVAM will initiate a dialogue with regulatory decision-makers from different sectors to discuss the potential role of ADME concepts for the 3Rs, and make the link to the EPAA.
- EPAA could invite industries to provide regulators with case studies to clarify/identify (minimal) regulatory requirements. This should include the use of ADME data to allow/support safety decision-making; proof-of-concept.
- EPAA could call industry members and other contributing parties to make use of the MEGen database and share data on selected compounds (e.g. form consortium)
- EPAA to assess if creating a “single portal” would be useful (ECVAM database, EPAA database, TSAR website etc.).
- Drafting of a decision paper by the ECHA (in preparation) on the testing proposal provided by companies which will then be distributed to national regulators for consultation. REACH provides more flexibility which might change mindset of regulators. ECHA has a very strong role in this process, whereas scientific committees (e.g. the SCCP) have a different, advisory role.

## 10. Conclusions

Although alternative ADME and toxicodynamics testing approaches have been used for decades, their application to safety testing strategies is of increasing importance, especially in light of new regulations with respect to chemical testing. It is recognised that the current *in vitro* metabolism models need improvement to offer more reliable information that is usable in safety assessment. To address this issue, an EPAA workshop was held in Duesseldorf in November, 2008, and brought together representatives from the pharmaceutical, chemical and cosmetic industries with those from (inter)national regulatory agencies. There are many alternative approaches used by different industrial sectors as compounds progress from identification to final products. A number of non-animal approaches not only allow for ethical testing but make good business sense in screening compounds for both efficacy and safety. The point at which animal tests come into safety assessment may be driven by regulations or by the lack of an *in vitro* model. Strategies that involve a small number of animals at early stages of development may also reduce the overall numbers of animal-based assays much later in development. Therefore refinement and reduction are evenly important challenges in the overall 3R target in the ADME area. *In vitro* systems that reflect certain aspects of the ADME (and effects) process can be very helpful in the safety assessment process as well as the 3R principal; but, on the other hand, many *in vitro* systems have their pitfalls, especially with respect to an insufficient reflection of the integrated *in vivo* physiological ADME conditions and a lack of fully validated assays. The recommendations proposed by representatives from different sectors and companies, which apply to all sectors, to propel the use of *in vitro* alternatives in the field of risk assessment are summarised below:

- Generate open web-based database on *in vivo* kinetic parameters.
- Industry data that are already available could be collected in a central database to benefit all sectors.
- Coordinate effort before generating new data.
- Expand Applicability Domains of *in silico* tools.
- Expand Applicability Domains of *in vitro* tools and develop new *in vitro* tools.
- Obligatory inclusion of blood sampling for TK purposes during toxicity studies.
- Investigate increased use of human low dose kinetics.
- Microdosing.
- Biomonitoring.

The workshop concluded that these assays still need to be improved but that it may be achieved by stakeholders from different sectors sharing data so that universal agreement is reached for harmonization of alternative approaches. Major international project funding programs are on-going to help develop, validate and harmonize *in vitro* tests and lead to their use as part of the risk assessment of chemicals.

## Conflict of interest

The authors of this article participated in the workshop organized and sponsored by EPAA, a partnership between industry and European Commission. Some of them received their travel expenses by the sponsor to make their participation in the workshop possible. If it seems necessary a list of those people who received travel expenses can be provided.

The employers of the authors are written in the affiliation list.

## Acknowledgements

The workshop was sponsored by EPAA (which sponsored the travel and accommodation of some participants from academia/regulatory bodies and financed the scientific writer) and by Henkel, as a member of EPAA (the workshop host).

## References

- Avdeef, A., Artursson, P., Neuhoof, S., Lazorova, L., Graso, J., Tavelin, S., 2005. Caco-2 permeability of weakly basic drugs predicted with the Double-Sink PAMPA pK(a)(flux) method. *European Journal of Pharmaceutical Sciences* 24, 333–349.
- Archer, G.E.B., Liebisch, M., 1998. The ECVAM international validation study *in vitro* for skin corrosivity. *Toxicology In Vitro* 12, 483–524.
- Bal-Price, A., Brown, G.C., 2001. Inflammatory neurodegeneration mediated by nitric oxide from activated glia-inhibiting neuronal respiration, causing glutamate release and excitotoxicity. *Journal of Neuroscience* 21, 6480–6491.
- Barton, H., Pastoor, T.P., Baetcke, K., Chambers, J.E., Diliberto, J., Doerrer, N.G., Driver, J.H., Hastings, C.E., Iyengar, S., Krieger, R., Stahl, B., Timchalk, C., 2006. The acquisition and application of absorption, distribution, metabolism, and excretion (ADME) data in agricultural chemical safety assessments. *Critical Reviews in Toxicology* 36, 9–35.
- Bi, Y.A., Kazolias, D., Duignan, D.B., 2006. Use of cryopreserved human hepatocytes in sandwich culture to measure hepatobiliary transport. *Drug Metabolism and Disposition* 34 (9), 1658–1665.
- Bjornsson, T.B., Callaghan, J.T., Einolf, H.J., Fischer, V., Gan, L., Grimm, S., Kao, J., King, S.P., Miwa, G., Ni, L., Kumar, G., McLeod, J., Obach, S.R., Roberts, S., Roe, A., Shah, A., Snikeris, F., Sullivan, J.T., Tweedie, D., Vega, J.M., Walsh, J., Wrighton, S.A., 2003. Pharmaceutical Research and Manufacturers of America Drug Metabolism/Clinical Pharmacology Technical Working Groups. The conduct of *in vitro* and *in vivo* drug–drug interaction studies: a PhRMA perspective. *Journal of Clinical Pharmacology* 43 (5), 443–469.
- Blaauboer, B.J., Boobis, A.R., Castell, J.V., Coecke, S., Groothuis, G.M.M., Guillouzo, A., Hall, T.J., Hawksworth, G.M., Lorenzon, G., Miltenburger, H.G., Rogiers, V., Skett, P., Villa, P., Wiebel, F.J., 1994. The practical applicability of hepatocyte cultures in routine testing. ECVAM Workshop Report No. 1. Alternatives to Laboratory Animals 22, 231–241.
- Blaauboer, B.J., 2003. The integration of data on physico-chemical properties, *in vitro*-derived toxicity data and physiologically based kinetic and dynamic as modelling a tool in hazard and risk assessment. A commentary. *Toxicology Letters* 138 (1–2), 161–171.
- Bogni, A., Monshouwer, M., Moscone, A., Hidestrand, M., Ingelman-Sundberg, M., Hartung, T., Coecke, S., 2005. Substrate specific metabolism by polymorphic cytochrome P450 2D6 alleles. *Toxicology In Vitro* 19 (5), 621–629.
- Boobis, A., Gundert-Remy, U., Kremers, P., Macheras, P., Pelkonen, O., 2002. In silico prediction of ADME and pharmacokinetics. Report of an expert meeting organised by COST B15. *European Journal of Pharmaceutical Sciences* 17 (4–5), 183–193.
- Bouvier d'Yvoire, M., Prieto, P., Blaauboer, B.J., Bois, F.Y., Boobis, A., Brochot, C., Coecke, S., Freidig, A., Gundert-Remy, U., Hartung, T., Jacobs, M.N., Lavé, T., Leahy, D.E., Lennernaes, H., Loizou, G.D., Meek, B., Pease, C., Rowland, M., Spendiff, M., Yang, J., Zeilmaker, M., 2007. Physiologically-based Kinetic Modelling (PBK Modelling): meeting the 3Rs agenda. The report and recommendations of ECVAM Workshop 63. Alternatives to Laboratory Animals 35, 661–671.
- Braissant, O., Henry, H., Villard, A.-M., Zurich, M.-G., Loup, M., Eilers, B., Parlascino, G., Matter, E., Boulat, O., Honegger, P., Bachmann, C., 2002. Ammonium-induced impairment of axonal growth is prevented through glial creatine. *Journal of Neuroscience* 22, 9810–9820.

- Braun, H., Buhneemann, C., Neumann, J., Reymann, K.G., 2006. Preparation of a tissue-like cortical primary culture from embryonic rats using Matrigel and serum free Start V Medium. *Journal of Neuroscience Methods* 15, 32–38.
- Bremer, S., Pellizzer, C., Coecke, S., Paparella, M., Catalani, P., 2002. Detection of the embryotoxic potential of cyclophosphamide by using a combined system of metabolic competent cells and embryonic stem cells. *Alternatives to Laboratory Animals* 30, 77–85.
- Bull, S., Langezaal, I., Clothier, R., Coecke, S., 2001. A genetically engineered cell-based system for detecting metabolism-mediated toxicity. *Alternatives to Laboratory Animals* 29, 703–716.
- Cai, H., Stoner, C., Reddy, A., Freiwald, S., Smith, D., Winters, R., Stankovic, C., Surendran, N., 2006. Evaluation of an integrated *in vitro*-in silico PBPK (physiologically based pharmacokinetic) model to provide estimates of human bioavailability. *International journal of pharmaceutics* 308 (1–2), 133–139.
- Chen, X., Murawski, A., Patel, K., Crespi, C.L., Balimane, P.V., 2008. A novel design of artificial membrane for improving the PAMPA model. *Pharmaceutical Research* 25 (7), 1511–1520.
- Coecke, S., Rogiers, V., Bayliss, M., Castell, J., Doehmer, J., Fabre, G., Fry, J., Kern, A., Westmoreland, C., 1999. The use of long-term hepatocyte cultures for detecting induction of drug metabolizing enzymes. The current status ECVAM hepatocytes and metabolically competent systems task force Report 1. *Alternatives to Laboratory Animals* 27, 579–638.
- Coecke, S., Bogni, A., Langezaal, I., Worth, A., Hartung, T., Monshouwer, M., 2001. The use of genetically engineered cells for assessing CYP2D6-related polymorphic effects. *Toxicology In Vitro* 15, 553–556.
- Coecke, S., Eskes, C., Gartion, J., Vliet, E.V., Kinsner, A., Bogni, A., Raimondo, L., Parisiss, N., Langezaal, I., 2002. Metabolism-mediated neurotoxicity: the significance of genetically engineered cell lines and new three-dimensional cell cultures. *Alternatives to Laboratory Animals* 30, 115–158.
- Coecke, S., Balls, M., Bowe, G., Davis, J., Gstraunthaler, G., Hartung, T., Hay, R., Merten, O.W., Price, A., Schechtman, L., Stacey, G., Stokes, W., 2005a. Second ECVAM Task Force on Good Cell Culture Practice. Guidance on good cell culture practice. A report of the second ECVAM task force on good cell culture practice. *Alternatives to Laboratory Animals* 33 (3), 261–287.
- Coecke, S., Blaauboer, B.J., Elaut, G., Freeman, S., Freidig, A., Gensmantel, N., Hoet, P., Kapoulas, V.M., Ladstetter, B., Langley, G., Leahy, D., Mannens, G., Meneguz, A., Monshouwer, M., Nemery, B., Pelkonen, O., Pfaller, W., Prieto, P., Proctor, N., Rogiers, V., Rostami-Hodjegan, A., Sabbioni, E., Steiling, W., van de Sandt, J.J., 2005b. Toxicokinetics and metabolism. *Alternatives to Laboratory Animals* 33 (1), 147–175.
- Coecke, S., Ahr, H., Blaauboer, B.J., Bremer, S., Casati, S., Castell, J., Combes, R., Corvi, R., Crespi, C.L., Cunningham, M.L., Elaut, G., Elett, B., Freidig, A., Gennari, A., Gheris-Egea, J.F., Guillouzo, A., Hartung, T., Hoet, P., Ingelman-Sundberg, M., Munn, S., Janssens, W., Ladstetter, B., Leahy, D., Long, A., Meneguz, A., Monshouwer, M., Morath, S., Nagelkerke, F., Pelkonen, O., Ponti, J., Prieto, P., Richert, L., Sabbioni, E., Schaack, B., Steiling, W., Testai, E., Vericat, J.A., Worth, A., 2006. Metabolism: a bottleneck in *in vitro* toxicological test development. The report and recommendations of ECVAM Workshop 54. *Alternatives to Laboratory Animals* 34 (1), 49–84.
- Coecke, S., Goldberg, A.M., Allen, S., Buzanska, L., Calamandrei, G., Crofton, K., Hareng, L., Hartung, T., Knauf, H., Honegger, P., Jacobs, M., Lein, P., Li, A., Mundy, W., Owen, D., Schneider, S., Silbergeld, E., Reum, T., Trnovec, T., Monnet-Tschudi, F., Bal-Price, A., 2007. Workgroup report: incorporating *in vitro* alternative methods for developmental neurotoxicity into international hazard and risk assessment strategies. *Environmental Health Perspectives* 115 (6), 924–931.
- Clemenson, C., Blaauboer, B., Castell, J., Prieto, P., Risteli, L., Vericat, J.A., Wendel, A., 2006. ACuteTox – optimisation and pre-validation of an *in vitro* test strategy for predicting human acute toxicity. *Alternativen zu Tierexperimenten* 23, 254–258.
- Clothier, R., Gottschalg, E., Casati, S., Balls, M., 2006. The FRAME alternatives laboratory database. 1. *In vitro* basal cytotoxicity determined by the Kenacid blue total protein assay. *Alternatives to Laboratory Animals* 34 (2), 151–175.
- Combes, R., Grindon, C., Cronin, M.T., Roberts, D.W., Garrod, J.F., 2008. Integrated decision-tree testing strategies for acute systemic toxicity and toxicokinetics with respect to the requirements of the EU REACH legislation. *Alternatives to Laboratory Animals* 36 (1), 91–109.
- Cooper-Hannan, R., Harbell, J.W., Coecke, S., Balls, M., Bowe, G., Cervinka, M., Clothier, R., Hermann, F., Klahm, L.K., de Lange, J., Liebsch, M., Vanparys, P., 1999. The principles of good laboratory practice: application to *in vitro* toxicology studies the report and recommendations of ECVAM Workshop 37. *Alternatives to Laboratory Animals* 27, 539–577.
- Cui, X., Thomas, A., Gerlach, V., White, R.E., Morrison, R.A., Cheng, K.C., 2008. Application and interpretation of hPXR screening data: validation of reporter signal requirements for prediction of clinically relevant CYP3A4 inducers. *Biochemical Pharmacology* 76 (5), 680–689.
- Creton, S., Billington, R., Davies, W., Dent, M.P., Hawksworth, G.M., Parry, S., Travis, K.Z., 2009. Application of toxicokinetics to improve chemical risk assessment: implications for the use of animals. *Regulatory Toxicology and Pharmacology* 55, 291–299.
- DeJongh, J., Nordin-Andersson, M., Ploeger, B.A., Forsby, A., 1999. Estimation of systemic toxicity of acrylamide by integration of *in vitro* toxicity data with kinetic simulations. *Toxicology and Applied Pharmacology* 158 (3), 261–268.
- Delic, J.L., Lilly, P.D., MacDonald, A.J., Loizou, G.D., 2000. The utility of PBPK in the safety assessment of chloroform and carbon tetrachloride. *Regulatory Toxicology and Pharmacology* 32 (2), 144–155.
- Dierickx, P.J., 2000. Cytotoxicity of the MEIC reference chemicals in rat hepatoma-derived Fa32 cells. *Toxicology* 150 (1–3), 159–169.
- Dierickx, P.J., Scheers, E.M., 2002. Neutral red uptake inhibition in adhered and adhering rat hepatoma-derived Fa32 cells to predict human toxicity. *Journal of Applied Toxicology* 22 (1), 61–65.
- Directive 76/768/EEC of 27 July 1976 on the approximation of the laws of the Member States relating to cosmetic products. *Official Journal of the European Communities* L262, 169–172.
- Directive 65/65/EEC, 1965. Council Directive 65/65/EEC of 26 January 1965 on the approximation of provisions laid down by law, regulation or administrative action relating to medicinal products. Available from: <[http://www.echamp.eu/fileadmin/user\\_upload/Regulation/Directive\\_65-65-EEC\\_-\\_Consolidated\\_Version.pdf](http://www.echamp.eu/fileadmin/user_upload/Regulation/Directive_65-65-EEC_-_Consolidated_Version.pdf)>.
- Dorne, J.L., Renwick, A.G., 2005. The refinement of uncertainty/safety factors in risk assessment by the incorporation of data on toxicokinetic variability in humans. *Toxicological Sciences* 86 (1), 20–26.
- Dorne, J.L., Walton, K., Slob, W., Renwick, A.G., 2002. Human variability in polymorphic CYP2D6 metabolism: is the kinetic default uncertainty factor adequate? *Food and Chemical Toxicology* 40 (11), 1633–1656.
- Dorne, J.L., Walton, K., Renwick, A.G., 2003. Polymorphic CYP2C19 and N-acetylation: human variability in kinetics and pathway-related uncertainty factors. *Food and Chemical Toxicology* 41 (2), 225–245.
- ECHA, 2009. ECHA/PR/09/11. New study inaccurate on the number of test animals for REACH. Available from: <[http://echa.europa.eu/doc/press/pr\\_09\\_11\\_animal\\_testing\\_20090828.pdf](http://echa.europa.eu/doc/press/pr_09_11_animal_testing_20090828.pdf)>.
- ECVAM, 2002. Biokinetics. *Alternatives to Laboratory Animals* 30 (1), 55–70.
- EFSA, 2007. Opinion of the Scientific Panel on Plant Protection Products and their Residues on a request from the Commission related to the revision of Annexes II and III to Council Directive 91/414/EEC concerning the placing of plant protection products on the market – toxicological and metabolism studies. 31 January 2007. Available from: <[http://www.efsa.europa.eu/EFSA/efsa\\_locale-1178620753812\\_1178620772682.htm](http://www.efsa.europa.eu/EFSA/efsa_locale-1178620753812_1178620772682.htm)>.
- EFSA Panel on Plant protection products and their Residues (PPR), 2007. Scientific Opinion related to the revision of Annexes II and III to Council Directive 91/414/EEC concerning the placing of plant protection products on the market: ecotoxicological studies, on request from the Commission. *EFSA Journal* 461, 1–44.
- Elsby, R., Surry, D.D., Smith, V.N., Gray, A.J., 2008. Validation and application of Caco-2 assays for the *in vitro* evaluation of development candidate drugs as substrates or inhibitors of P-glycoprotein to support regulatory submissions. *Xenobiotica* 38 (7–8), 1140–1164.
- ESAC, 2001. Statement on the scientific validity of the Embryonic Stem Cell Test (EST), the Micromass Test and the Post implantation Rat Whole-Embryo Culture Assay – *in vitro* tests for embryo toxicity. ECVAM Scientific Advisory Committee (ESAC), 17th Meeting, 16–17 October, Ispra, Italy.
- ESAC, 2008. Statement on the Scientific Validity of In-vitro Tests for Skin Irritation Testing. 5th November 2008. Available from: <http://ecvam.jrc.it/index.htm>.
- Eskes, C., Zuang, V., 2005. Alternative (non-animal) methods for cosmetics testing: current status and future prospects. *Alternatives to Laboratory Animals* 33 (1), 47–81 (Chapter 9).
- EU, 1976. Council Directive 76/768/EEC of 27 July 1976 on the approximation of the laws of the Member States relating to cosmetic products. *Official Journal* L262, 169–200.
- EU, 1993. Council Directive 93/35/EEC of 14 June 1993 amending for the sixth time Directive 76/768/EEC on the approximation of the laws of the Member States relating to cosmetic products. *Official Journal* L151, 32–37.
- EU, 2010. Regulation (EC) No. 1107/2009 of the European Parliament and of the Council of 21 October 2009 concerning the placing of plant protection products on the market and repealing Council Directives 79/117/EC and 91/414/EEC.
- Evans, W.E., Relling, M.V., 1999. Pharmacogenomics: translating functional genomics into rational therapeutics. *Science* 286 (5439), 487–491.
- FDA, 2005. Guidance for Industry ANDAs: Impurities in Drug Substances.
- FDA, 2006. Food and Drug Administration (FDA): Drug Interaction Studies – Study Design, Data Analysis, and Implications for Dosing” 2006. Available from: <<http://www.fda.gov/cder/Guidance/6695dft.pdf>>.
- FDA, 2008. Food and Drug Administration (FDA): Guidance for Industry Safety Testing of Drug Metabolites, February, 2008. Available from: <<http://www.fda.gov/CDER/GUIDANCE/6897n1.pdf>>.
- Garberg, P., Ball, M., Borg, N., Cecchelli, R., Fenart, L., Hurst, R.D., Lindmark, T., Mabondzo, A., Nilsson, J.E., Raub, T.J., Stanimirovic, D., Terasaki, T., Oberg, J.O., Osterberg, T., 2005. *In vitro* models for the blood–brain barrier. *Toxicology In vitro* 19 (3), 299–334.
- Goebel, C., Hewitt, N.J., Kunze, G., Wenker, M., Hein, D.W., Beck, H., Skare, J., 2009. Skin metabolism of aminophenols: human keratinocytes as a suitable *in vitro* model to qualitatively predict the dermal transformation of 4-amino-2-hydroxytoluene *in vivo*. *Toxicology and Applied Pharmacology* 235 (1), 114–123.
- Guillouzo, A., 1998. Liver cell models in *in vitro* toxicology. *Environmental Health Perspectives* 106 (Suppl. 2), 511–532 (Review).
- Harmsen, S., Koster, A.S., Beijnen, J.H., Schellens, J.H., Meijerman, I., 2008. Comparison of two immortalized human cell lines to study nuclear receptor-mediated CYP3A4 induction. *Drug Metabolism and Disposition* 36 (6), 1166–1171.
- Hartung, T., Rovida, C., 2009. Chemical regulators have overreached. *Nature* 460 (7259), 1080–1081.

- Hayashi, M., Kamat, E., Hirose, A., Takahashi, M., Morita, T., Ema, M., 2005. *In silico* assessment of chemical mutagenesis in comparison with results of Salmonella microsome assay on 909 chemicals. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis* 588 (2), 129–135.
- Hewitt, N.J., Hewitt, P., 2004. Phase I and II enzyme characterization of two sources of HepG2 cell lines. *Xenobiotica* 34 (3), 243–256.
- Hong, M.S., Hong, S.J., Barhoumi, R., Burghardt, R.C., Donnelly, K.C., Wild, J.R., Venkatraj, V., Tiffany-Castiglioni, E., 2003. Neurotoxicity induced in differentiated SK-N-SH-SY5Y human neuroblastoma cells by organophosphorus compounds. *Toxicology and Applied Pharmacology* 186, 110–118.
- Horizontal Legislation, 2008. EU Environmental Policy Handbook: Providing Tools to Achieve Environmental Objectives. Available from: <[http://ec.europa.eu/competition/state\\_aid/legislation/horizontal.html](http://ec.europa.eu/competition/state_aid/legislation/horizontal.html)>.
- Hubatsch, I., Ragnarsson, E.G., Artursson, P., 2007. Determination of drug permeability and prediction of drug absorption in Caco-2 monolayers. *Nature Protocols* 2, 2111–2119.
- ICRP, 1991. ICRP 62. Radiological protection in biomedical research, Ann. ICRP 22(3), 1991. P 062 erratum in P 075 JAICRP 27(1).
- ICRP, 2007. Publication 103. The recommendations of the international commission on radiological protection. In: Valentin, J. (Ed.), *Annals of the ICRP*. Elsevier, pp. 1–34.
- Irvine, J.D., Takahashi, L., Lockhart, K., Cheong, J., Tolan, J.W., Selick, H.E., Grove, J.R., 1999. MDCK (Madin–Darby canine kidney) cells: a tool for membrane permeability screening. *Journal of Pharmaceutical Sciences* 88 (1), 28–33.
- Janmohamed, A., Dolphin, C.T., Phillips, I.R., Shephard, E.A., 2001. Quantification and cellular localization of expression in human skin of genes encoding flavin-containing monooxygenases and cytochromes P450. *Biochemical Pharmacology* 62 (6), 777–786.
- Jennings, P., Aydin, S., Bennett, J., McBride, R., Weiland, C., Tuite, N., Gruber, L.N., Perco, P., Gaora, P.O., Ellinger-Zieglerbauer, H., Ahr, H.-J., Van Kooten, C., Dahi, M.R., Prieto, P., Ryan, M.P., Pfaller, W., McMorro, T., 2009. Inter-laboratory comparison of human renal proximal tubule (HK-2) transcriptome alterations due to Cyclosporine A exposure and medium exhaustion. *Toxicology in Vitro* 23 (3), 486–499.
- Kinsner-Ovaskainen, A., Akkan, Z., Casati, S., Coecke, S., Corvi, R., Dal Negro, G., De Bruijn, J., De Silva, O., Gribaldo, L., Griesinger, C., Jaworska, J., Kreysa, J., Maxwell, G., McNamee, P., Price, A., Prieto, P., Schubert, R., Tosti, L., Worth, A., Zuang, V., 2009. Overcoming barriers to validation of non-animal partial replacement methods/Integrated Testing Strategies: the report of an ECVAM-ECVAM workshop. *Alternatives to Laboratory Animals* 37 (4), 437–444.
- Kirkland, D., Aardema, M., Henderson, L., Mueller, L., 2005. Evaluation of the ability of a battery of three *in vitro* genotoxicity tests to discriminate rodent carcinogens and non-carcinogens I. Sensitivity, specificity and relative predictivity. *Mutation Research* 584 (1–2), 1–256. Erratum in: *Mutation Research* (2005), 588(1), 70.
- Kneuer, C., Lakoma, C., Honscha, W., 2007. Prediction of acute toxicity in HPCT-1E3 hepatocytoma cells with liver-like transport activities. *Alternatives to Laboratory Animals* 35 (4), 411–420.
- Kollock, R., Frank, H., Seidel, A., Meini, W., Glatt, H., 2008. Oxidation of alcohols and reduction of aldehydes derived from methyl- and dimethylpyrenes by cDNA-expressed human alcohol dehydrogenases. *Toxicology* 245 (1–2), 65–75.
- LeBoeuf, R.A., Kerckaert, G.A., Aardema, M.J., Gibson, D.P., Brauning, R., Isfort, R.J., 1996. The pH 6.7 Syrian hamster embryo cell transformation assay for assessing the carcinogenic potential of chemicals. *Mutation Research* 356 (1), 85–127.
- Lehman, A.J., Fitzhugh, O.G., 1954. 100-fold margin of safety. *Association of Food and Drug Officials' USQ Bulletin* 18, 33–35.
- Liang, M., Ramsey, C.R., Knox, F.G., 1999. The paracellular permeability of opossum kidney cells, a proximal tubule cell line. *Kidney International* 56 (6), 2304–2308.
- MacGregor, J.T., Collins, J.M., Sugiyama, Y., Tyson, C.A., Dean, J., Smith, L., Andersen, M., Curren, R.D., Houston, J.B., Kadlubar, F.F., Kedderis, G.L., Krishnan, K., Li, A.P., Parchment, R.E., Thummel, K., Tomaszewski, J.E., Ulrich, R., Vickers, A.E., Wright, S.A., 2001. *In vitro* human tissue models in risk assessment: report of a consensus-building workshop. *Toxicological Sciences* 59 (1), 17–36.
- McGee, P., 2006. The assay validation challenge. *Drug Discovery & Development*. Available from: <<http://www.dddmag.com/the-assay-validation-challenge.aspx>>.
- McGinnity, D.F., Riley, R.J., 2001. Predicting drug pharmacokinetics in humans from *in vitro* metabolism studies. *Biochemical Society Transactions* 29 (2), 135–139.
- Maeshima, H., Ohno, K., Tanaka-Azuma, Y., Nakano, S., Yamada, T., 2009. Identification of tumor promotion marker genes for predicting tumor promoting potential of chemicals in BALB/c 3T3 cells. *Toxicology in Vitro* 23 (1), 148–157.
- Munn, G.C., Aardema, M.J., Hu, T., Barnett, B., Kaluzhny, Y., Klausner, M., Karetzky, V., Dahl, E.L., Curren, R.D., 2009. Further development of the EpiDerm™ 3D reconstructed human skin micronucleus (RSMN) assay. *Mutation Research* 673, 92–99.
- OECD TG 428 – OECD guideline for the testing of chemicals. Skin absorption: *in vitro* method. 428. Adopted: 13 April 2004.
- OECD, 2004. Advisory Document 14 of the OECD Working Group on GLP: The Application of the Principles of GLP to *in vitro* Studies. ECVAM & ICCVAM.
- OECD TG 430 – OECD guideline for testing of chemicals – Draft Guideline 430: *In vitro* skin corrosion: transcutaneous electrical resistance test (TER), organization for economic cooperation and development. Adopted: 13 April 2004.
- OECD TG 471 – OECD Guidelines for the Testing of Chemicals, No. 471: Bacterial Reverse Mutation Test. Adopted: 26 May 1983, up-dated: 21 July 1997.
- OECD TG 473 – OECD Guidelines for the Testing of Chemicals, No. 473: *In vitro* Mammalian Chromosome Aberration Test. Adopted: 26 May 1983, up-dated: 21 July 1997.
- OECD TG 495 – OECD Guideline for testing of chemicals, No. 495 DRAFT: *In vitro* Syrian Hamster Embryo (SHE) Cell Transformation Assay. Organization for Economic Cooperation and Development, Paris, draft approved.
- Oesch, F., Fabian, E., Oesch-Bartlomowicz, B., Werner, C., Landsiedel, R., 2007. Drug-metabolizing enzymes in the skin of man, rat, and pig. *Drug Metabolism Reviews* 39 (4), 659–698.
- Pauwels, M., Rogiers, V., 2004. Safety evaluation of cosmetics in the EU. Reality and challenges for the toxicologist. *Toxicology Letters* 151 (1), 7–17.
- Pawliszyn, J., 1995. New directions in sample preparation for analysis of organic compounds. *Trends in Analytical Chemistry* 14 (3), 113–122.
- Pelkonen, O., Myllynen, P., Taavitsainen, P., Boobis, A.R., Watts, P., Lake, B.G., Price, R.J., Renwick, A.B., Gómez-Lechón, M.J., Castell, J.V., Ingelman-Sundberg, M., Hidesbrand, M., Guillouzo, A., Corcos, L., Goldfarb, P.S., Lewis, D.F., 2001. Carbamazepine: a 'blind' assessment of CYP-associated metabolism and interactions in human liver-derived *in vitro* systems. *Xenobiotica* 31 (6), 321–343.
- Pelkonen, O., Turpeinen, M., Uusitalo, J., Rautio, A., Raunio, H., 2004. Prediction of drug metabolism and interactions on the basis of *in vitro* investigations. *Basic & Clinical Pharmacology & Toxicology* 96, 167–175.
- Plant, N., 2004. Strategies for using *in vitro* screens in drug metabolism. *Drug Discovery Today* 9 (7), 328–336.
- Prieto, P., Blaauwboer, B.J., de Boer, A.G., Boveri, M., Cecchelli, R., Clemenson, C., Coecke, S., Forsby, A., Galla, H.J., Garberg, P., Greenwood, J., Price, A., Taehiti, H., 2004. Blood–brain barrier *in vitro* models and their application in toxicology. *Alternatives to Laboratory Animals* 32, 37–50.
- Renwick, A.G., Lazarus, N.R., 1998. Human variability and noncancer risk assessment – an analysis of the default uncertainty factor. *Regulatory Toxicology and Pharmacology* 27 (1 Pt. 2), 3–20.
- Rogiers, V., Pauwels, M., 2008. Safety Assessment of Cosmetics in Europe. *Current Problems in Dermatology*, vol. 37. S. Karger, Basel, CH, ISBN 978-3-8055-8655-9.
- Saliner, A.G., Tsakovska, I., Pavan, M., Patlewicz, G., Worth, A.P., 2007. Evaluation of SARs for the prediction of skin irritation/corrosion potential: structural inclusion rules in the BfR decision support system. *SAR QSAR in Environmental Research* 18 (3–4), 331–342.
- SANCO, 2004. Guidance Document on Dermal Absorption (Sanco/222/2000 rev.7, 19 March 2004) European Commission, Health & Consumer Protection Directorate-General. Available from: <[http://ec.europa.eu/food/plant/protection/evaluation/guidance/wrkd020\\_rev\\_en.pdf](http://ec.europa.eu/food/plant/protection/evaluation/guidance/wrkd020_rev_en.pdf)>.
- Sanderson, D.M., Earnshaw, C.G., 1991. Computer prediction of possible toxic action from chemical structure; the DEREK system. *Human and Experimental Toxicology* 10, 261–273.
- SCCP/1005/06: The SCCP's Notes of Guidance for the Testing of Cosmetic Ingredients and their Safety Evaluation, adopted by the SCCP during the 10th plenary meeting of 19 December 2006.
- SCCS/1358/10: SCCS (Scientific Committee on Consumer Safety). Basic criteria for the *in vitro* assessment of dermal absorption of cosmetic ingredients. The SCCS adopted this opinion at its 7th plenary meeting of 22 June 2010.
- Shou, M., Hayashi, M., Pan, Y., Xu, Y., Morrissey, K., Xu, L., Skiles, G.L., 2008. Modeling, prediction, and *in vitro* *in vivo* correlation of CYP3A4 induction. *Drug Metabolism and Disposition* 36 (11), 2355–2370.
- Schnackenberg, L.K., Chen, M., Sun, J., Holland, R.D., Dragan, Y., Tong, W., Welsh, W., Beger, R.D., 2008. Evaluations of the trans-sulfuration pathway in multiple liver toxicity studies. *Toxicology and Applied Pharmacology* [Epub ahead of print].
- Skare, J.A., Hewitt, N.J., Doyle, E., Powrie, R., Elcombe, C., 2009. Metabolite screening of aromatic amine hair dyes using *in vitro* hepatic models. *Xenobiotica* 39 (11), 811–825.
- Spielmann, H., Balls, M., Dupuis, J., Pape, W.J.W., De Silva, O., Holzhuetter, H.G., Gerberich, F., Liebsch, M., Lowell, W.W., Pfannenbecker, V., 1998. A study on UV filter chemicals from Annex VII of the European Union Directive 76/768/EEC in the *in vitro* NRU Phototoxicity test. *Alternatives to Laboratory Animals* 26, 679–708.
- Spielmann, H., Hoffmann, S., Liebsch, M., Botham, P., Fentem, J.H., Eskes, C., Roguet, R., Cotovio, J., Cole, T., Worth, A., Heylings, J., Jones, P., Robles, C., Kandarova, H., Gamer, A., Remmele, M., Curren, R., Raabe, H., Cockshott, A., Gerner, I., Zuang, V., 2007. The ECVAM international validation study on *in vitro* tests for acute skin irritation: report on the validity of the EPISKIN and EpiDerm assays and on the Skin Integrity Function Test. *Alternatives to Laboratory Animals* 35 (6), 559–601.
- Thomas, S., Brightman, F., Gill, H., Lee, S., Pufong, B., 2008. Simulation modelling of human intestinal absorption using Caco-2 permeability and kinetic solubility data for early drug discovery. *Journal of Pharmaceutical Sciences* 97 (10), 4557–4574.
- Tox21, 2008. Available from: <[http://www.epa.gov/comptox/articles/comptox\\_mou.html](http://www.epa.gov/comptox/articles/comptox_mou.html)>.
- Truhaut, R., 1991. The concept of the acceptable daily intake: an historical review. *Food Additives and Contaminants* 8, 151–162.
- Uehara, T., Kiyosawa, N., Shimizu, T., Omura, K., Hirode, M., Imazawa, T., Mizukawa, Y., Ono, A., Miyagishima, T., Nagao, T., Urushidani, T., 2008. Species-specific differences in coumarin-induced hepatotoxicity as an example toxicogenomics-based approach to assessing risk of toxicity to humans. *Human and Experimental Toxicology* 27 (1), 23–35.

- Vaes, W.H.J., Urrestarazu-Ramos, E., Verhaar, H.J.M., Seinen, W., Hermens, J.L.M., 1996. Measurement of the free concentration using solid-phase microextraction: binding to protein. *Analytical Chemistry* 68, 4463–4467.
- Van Leeuwen, C.J., 2007. General introduction. In: Van Leeuwen, C.J., Vermeire, T.G. (Eds.), *Risk Assessment of Chemicals; An Introduction*, second ed. Springer, Dordrecht, pp. 1–36.
- Vignati, L., Turlizzi, E., Monaci, S., Grossi, P., Kanter, R., Monshouwer, M., 2005. An *in vitro* approach to detect metabolite toxicity due to CYP3A4-dependent bioactivation of xenobiotics. *Toxicology* 216 (2–3), 154–167.
- Westerink, W.M., Schoonen, W.G., 2007a. Cytochrome P450 enzyme levels in HepG2 cells and cryopreserved primary human hepatocytes and their induction in HepG2 cells. *Toxicology In Vitro* 21 (8), 1581–1591.
- Westerink, W.M., Schoonen, W.G., 2007b. Phase II enzyme levels in HepG2 cells and cryopreserved primary human hepatocytes and their induction in HepG2 cells. *Toxicology In Vitro* 21 (8), 1592–1602.
- Wieser, M., Stadler, G., Jennings, P., Streubel, B., Pfaller, W., Ambros, P., Riedl, C., Katinger, H., Grillari, J., Grillari-Voglauer, R., 2008. HTERT alone immortalizes epithelial cells of renal proximal tubules without changing their functional characteristics. *American Journal of Physiology. Renal Physiology* 295 (5), F1365–F1375.
- Zuber, R., Anzenbacherova, E., Anzenbacher, P., 2002. Cytochromes P450 and experimental models of drug metabolism. *Journal of Cellular and Molecular Medicine* 6 (2), 189–198.